Signaling Mechanisms in the Vertebrate Retina

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"Research is formalized curiosity. It is poking and prying with a purpose."

Zora Neale Hurston

Summary

Image-forming vision of vertebrates is mediated by the retina, which collects visual information and performs sophisticated computations before conveying the output to higher brain areas. In this thesis, I made use of the advantages offered by the vertebrate model organism zebrafish to gain new insights into retinal function. Parallel processing of visual stimulus properties is fundamental to vision. Besides photoreceptors, the retina contains a number of neuronal cell types processing the visual signal. Bipolar cells, located in the retinal inner nuclear layer (INL), have a central position in the retinal connectome, being the only cell type contacted by all other classes of retinal neurons. Distinct bipolar cell subtypes are thought to constitute parallel channels for properties of a visual stimulus. For example, the group of ON bipolar cells hyperpolarizes in response to glutamate, while OFF bipolar cells depolarize. Although our knowledge of bipolar cell function has greatly advanced, it is largely unknown how individual subtypes shape the visual signal. Transgenic techniques are powerful tools to study labelled neurons and gain insight into their roles in retinal processing. In order to establish transgenic lines with different populations of bipolar cells labelled, we have identified putative regulatory regions of genes expressed in bipolar cells and tested their ability to drive expression in bipolar cells. We show that *cabp2a*, *cabp2b*, *cabp5a* and *cabp5b*, encoding Ca^{2+}-binding proteins, are specifically expressed in the INL of developing and adult zebrafish, and that regulatory regions of *cabp2a*, *cabp5a* and *cabp5b* drive reporter expression in bipolar cells. Another interesting expression pattern is found in the *mglur6b:EGFP* line, where a regulatory region of *mglur6b*, involved in the ON bipolar cell response, drives reporter expression. By investigating *mglur6b:EGFP* expression in detail, we extend knowledge of mglur6 expression to specific cell types such as olfactory bulb projection neurons, suggesting novel functions of mGluR6 signaling in the brain.

In addition, I have investigated the role of auxiliary $\alpha_2\delta_4$ subunits of voltage-gated Ca^{2+} channels. Mutations in *CACNA2D4*, encoding human $\alpha_2\delta_4$, cause retinal defects affecting mainly the cone system. The cone-dominant zebrafish is an excellent model to study onset and progression of this disease. We have identified two zebrafish orthologues to human *CACNA2D4*, termed *cacna2d4a* and *cacna2d4b*, and demonstrate conserved expression patterns in photoreceptors and the INL. After CRISPR/Cas9 mediated knockout of *cacna2d4b*, we observed downregulation of the pore-forming Ca^{2+} channel subunit Cacna1fa at photoreceptor terminals. This effect seems to be very specific, since retinal morphology and localization of other synaptic proteins investigated were unaffected. Since conserved roles of genes encoding $\alpha_2\delta_4$ in trafficking of Ca^{2+} channel complexes are thus suggested, this phenotype provides a good starting point for studying the potentially progressive disease character in zebrafish.

The common ancestor of all extant teleost fishes has undergone whole genome duplication around 350 million years ago. While most duplicated genes have been lost in the lineage leading to zebrafish, up to 20% of duplicated genes have been retained, including disproportionately high numbers of genes with neuronal function among them. One chapter of this thesis summarizes and discusses consequences of the teleost-specific whole genome duplication event.

Zusammenfassung

Das Sehvermögen von Vertebraten wird durch die Retina vermittelt, die Lichtinformation sammelt und aufwendig verrechnet, bevor sie das visuelle Signal an übergeordnete Gehirnzentren weitervermittelt. In dieser Arbeit zog ich Nutzen aus den Vorteilen des Vertebraten-Modellorganismus Zebrafisch, um neue Erkenntnisse über Funktionen der Retina zu gewinnen.

Ein grundlegendes Prinzip des Sehens ist parallele Verarbeitung von Eigenschaften eines visuellen Stimulus. Neben Photorezeptoren enthält die Retina auch einige neuronale Zelltypen die das visuelle Signal verarbeiten. Bipolarzellen, die in der inneren Körnerschicht liegen, spielen eine zentrale Rolle im Netzwerk der Retina, weil sie der einzige Zelltyp sind der von allen anderen retinalen Zelltypen kontaktiert wird. Es wird angenommen, dass unterschiedliche Subtypen von Bipolarzellen parallele Kanäle für Eigenschaften eines visuellen Stimulus bereitstellen. Zum Beispiel hyperpolarisieren ON Bipolarzellen als Antwort auf Glutamat, während OFF Bipololarzellen depolarisieren. Obwohl unser Wissen über die Funktionen von Bipolarzellen grosse Fortschritte gemacht hat, ist immer noch nicht bekannt, wie einzelne Subtypen das visuelle Signal formen. Transgene Techniken stellen wirkungsvolle Werkzeuge dar um markierte Bipolarzellen zu erforschen, und um tiefere Einblicke in ihre Rollen in der Signalverarbeitung zu gewinnen. Um transgene Linien zu schaffen, identifizierten wir mutmassliche regulatorische Regionen von Genen die von Bipolarzellen exprimiert werden und testeten ihre Fähigkeit Expression in Bipolarzellen zu vermitteln. Wir konnten zeigen, dass *cabp2a*, *cabp2b*, *cabp5a* und *cabp5b*, Gene die für calciumbindende Proteine codieren, spezifisch in Bipolarzellen exprimiert werden, und dass genomische regulatorische Sequenzen von *cabp2a*, *cabp5a* und *cabp5b* die Expression von Reportergenen in Bipolarzellen aktivieren. Anhand der *mglur6b:EGFP* transgenen Linie fanden wir ein weiteres interessantes Expressionsmuster. In dieser Linie kontrollieren regulatorische Sequenzen von *mglur6b*, das an der ON Antwort von Bipolarzellen beteiligt ist, Expression des Reportergens. Indem wir *mglur6b:EGFP transgene* Zebrafische genau untersuchten, konnten wir das Wissen über *mglur6* Expression auf bestimmte Zelltypen, wie Output Neuronen des Riechkolbens, erweitern, was neuartige Funktionen von Signalvermittlung durch mGluR6 impliziert.

Zusätzlich untersuchte ich die Rolle von auxiliären $\alpha_2\delta_4$ Calciumkanaluntereinheiten. Mutationen in *CACNA2D4*, das für $\alpha_2\delta_4$ des Menschen codiert, verursachten Defekte der Retina die hauptsächlich das zapfenvermittelte Sehen betreffen. Die zapfendominierte Retina des Zebrafisches ist ein exzellentes Modellsystem um Einsetzen und Fortschreiten dieser Erkrankung zu untersuchen. Wir identifizierten zwei Orthologe zu menschlichem *CACNA2D4*, die mit *cacna2d4a* und *cacna2d4b* benannt sind, und stellten konservierte Expressionsmuster in Photorezeptoren und der inneren Körnerschicht fest. Infolge CRISPR/Cas9 vermittelten Knockouts von *cacna2d4b* konnte Herabregulierung der kanalbildenden Calciumkanaluntereinheit Cacna1fa an der Photorezeptorsynapse beobachtet werden. Dieser Effekt scheint sehr spezifisch zu sein, weil weder Morphologie der Retina noch Lokalisation anderer untersuchter synaptischer

Proteine verändert waren. Da deshalb eine konservierte Funktionen von $\alpha_2\delta_4$ Proteinen im Trafficking von Calciumkanalkomplexen impliziert wird, stellt dieser Phänotyp einen guten Ausgangspunkt für die Untersuchung des potentiell progressiven Krankheitsverlaufs dar.

Vor etwa 350 Millionen Jahren erfuhr der gemeinsame Vorfahr aller rezenten Teleostei eine Genomduplikation. Obwohl die meisten duplizierten Gene in der Abstammungslinie des Zebrafisches wieder verloren gingen, blieben bis zu 20% der Gene als Duplikate erhalten. Ein disproportional hoher Anteil dieser Gen-Paare hat neuronale Funktion. Ein Kapitel dieser Arbeit befasst sich mit den Konsequenzen dieser Genomduplikation.

Contents

Chapter 1

General Introduction

Image-forming vision of vertebrates is mediated by the retina. This constitutes not only light gathering, but also sophisticated processing of visual information before conveying information to higher brain centers. The retina covers the inner surface of the eye, and although located in the periphery outside of the skull, is part of the central nervous system. During development, the optic vesicles bulge out from the diencephalon, eventually giving rise to the fully developed retina that contains light sensitive cells as well as several neuronal cell types for processing visual information (Figure 1.1A).

1.1 Signaling in the vertebrate retina

Two types of photoreceptors, situated in the most distal (or outermost) retinal layer, can be found in the retinae of most vertebrate species, the cones that mediate day-light (photopic) vision and the very light-sensitive rods important for vision at low luminance levels (scotopic vision). Photoreceptors detect light by visual pigments, consisting of a light-absorbing chromophore coupled to one of several different opsins, tuning sensitivity of the visual pigment to different wavelengths. Absorption a photon triggers the phototransduction cascade that ultimately leads to hyperpolarization of the photoreceptor.

Two major directions of information flow exist for transmitting and processing visual information detected by photoreceptors (Figure 1.1A). In the vertical pathway, information is transmitted from photoreceptors to bipolar cells and from bipolar cells to retinal ganglion cells. The axons of ganglion cells converge to form the optic nerve, communicating the retinal output to other brain regions. Horizontal information flow serves to execute computations within the two synaptic layers of the retina. Horizontal cells, situated in the distal inner nuclear layer, contribute to retinal processing in the outer plexiform layer, while amacrine cells, lying in the most proximal inner nuclear layer (INL), modify signals transmitted from bipolar to ganglion cells in the inner plexiform layer.

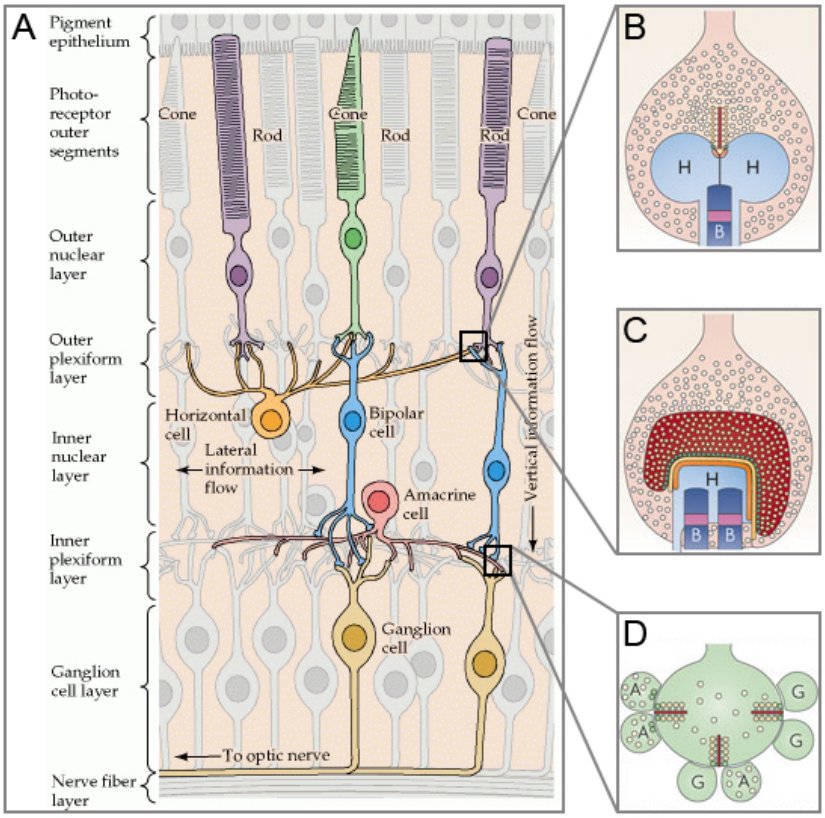

FIGURE 1.1: **Structure of the vertebrate retina. (A)** Two photoreceptor types (rods and cones) are found in the most distal part of the retina. The inner nuclear layer is populated by horizontal and bipolar cells contacting each other and photoreceptors in the outer plexiform layer, and by amacrine cells connecting to bipolar cells and ganglion cells in the inner plexiform layer. **(B,C,D)** Photoreceptors and bipolar cells are characterized by specialized presynaptic ribbons. Although both rods and cones carry ribbon synapses, only the rod synaptic terminal is shown for simplicity. En-face view **(C)** of the ribbon reveals its horse shoe-like shape. In **(B-D)**, synaptic ribbons are shown in dark red, AMPA receptors in orange and the arciform density in yellow. Vesicles attached to ribbon are represented by yellow spheres and docked vesicles by green spheres. A, amacrine cell process; B, ON bipolar cell dendrite; G, ganglion cell dendrite; H, horizontal cell dendrite. (B-D) adapted from Purves, (2012); (B-D) adapted from Matthews and Fuchs, (2010).

1.2 Synaptic transmission from retinal ribbon synapses

Synaptic transmission from photoreceptors and bipolar cells is unusual in the sense that these cells transmit graded information, in contrast to all-or-nothing signaling found at most other synapses. To fulfill this role, photoreceptors and bipolar cells of the retina, as well as a few other cell types (pineal photoreceptors, electroreceptors, hair cells of the inner ear and lateral line organ), have evolved specialized structures termed ribbon synapses. Ribbon synapses are marked by an electron-dense, elongated structure, termed the ribbon, having numerous glutamate-filled vesicles tethered. Ribbon synapses have been recognized as important for increasing the amount of vesicles available to ensure sustained glutamate release, and may also have important roles as a transport system for synaptic vesicles and in synchronized release of multiple vesicles when needed (reviewed by Matthews and Fuchs, 2010).

The photoreceptor synaptic ribbons (Figure 1.1B,C) are large, horseshoe-shaped planar structures (Figure 1.1C) that exhibit their typical elongated, ribbon-like appearance when cut transversally (Figure 1.1B). In rods, ribbons can run several microns along the emarginated photoreceptor terminal and have thousands of synaptic vesicles tethered, a requirement probably posed by the fact that hundreds of vesicles can be released from a photoreceptor synapse in only one second (Heidelberger, Thoreson, and Witkovsky, 2005).

At the base of the photoreceptor ribbon, another electron-dense structure, the arciform density is found, anchoring the ribbon to the plasma membrane (Figure 1.1B,C). This is mediated by interaction of Ribeye, the main structural component of the ribbon, with Bassoon, an important component of the arciform density (Tom Dieck et al., 2005). The voltage-gated Ca^{2+} channels mediating synaptic vesicle exocytosis are located in the plasma membrane in close proximity to the arciform density, arranged as an array in the plasma membrane, running along the ribbon.

Ribbons at bipolar cell synaptic terminals (Figure 1.1D) are smaller than photoreceptor ribbons, each having only around 100 vesicles tethered. This smaller pool of vesicles probably relates to the more transient release of glutamate from bipolar cells compared to photoreceptors. However, each bipolar cell contains from 30 to more than 100 ribbons, depending on species and subtype of bipolar cell (reviewed by Matthews and Fuchs, 2010. This high number of ribbons illustrates the complex connectivity of each bipolar cell with numerous ganglion and amacrine cells in the inner plexiform layer (IPL).

1.3 Separation of the visual signal into ON and OFF channels

Parallel processing of properties of a visual stimulus, like contrast information, chromatic composition, movement or temporal profile, is fundamental to vision (Euler et al., 2014; Wassle, 2004). The separation of visual information into different channels already starts at the first retinal synapse, where photoreceptors contact different types of bipolar cells. The

separation in ON and OFF channels is crucial for contrast vision, and is achieved through differential expression of glutamate receptors by ON and OFF types of bipolar cells. Glutamate, released from depolarized photoreceptors in darkness, binds to ionotropic AMPA/kainite glutamate receptors on the dendritic tips of OFF bipolar cells, leading to channel opening and therefore depolarization. In contrast, ON bipolar cells sense glutamate via the inhibitory metabotropic glutamate receptor mGluR6, triggering closure of the cation conducting TRPM1 channel by G-protein signaling (Koike et al., 2010; Morgans et al., 2009). Closure of this unselective cation channel results in hyperpolarization of the ON bipolar cell, and therefore in reversal of the photoreceptor signal. In addition to mGluR6 signaling, another mechanism for the hyperpolarizing response of ON bipolar cells to glutamate has been discovered. Excitatory amino acid transporters (EAATs), mostly known for removing glutamate from the synaptic cleft, most probably also contribute to ON signaling by activation of a Cl^- conductance upon glutamate binding. This mechanism has been described in fish (Wong, Adolph, and Dowling, 2005; Wong, Cohen, and Dowling, 2005; Wong and Dowling, 2005; Wong et al., 2004; Grant and Dowling, 1995; Grant and Dowling, 1996; Nawy and Copenhagen, 1987), but might also contribute to the ON response in mammals (Tse, Chung, and Wu, 2014).

1.4 Bipolar cell subtypes in visual processing

As the only neurons in the retina contacting every other retinal cell type, bipolar cells play a central role in processing of the visual signal. Several subtypes of bipolar cells exist, and each is thought to constitute a separate channel for transmitting visual stimulus properties (Asari and Meister, 2012; Euler et al., 2014). In addition to separation into ON and OFF channels, other mechanisms have been recognized as important for creating distinct channels through the retina. Bipolar cells contact multiple photoreceptors, and at the same time, each photoreceptor is contacted by several subtypes of bipolar cells (Li et al., 2012; Wassle et al., 2009). Preferentially contacting certain photoreceptor types creates distinct channels for transmitting chromatic information. Recently, the size of a bipolar cell axon terminal has been recognized as a factor in temporal filtering. While bigger axon terminals transmit signals more slowly, small terminals are faster and can resolve higher stimulus frequencies better (Baden et al., 2014). In rodents, 13 bipolar cell subtypes have been identified based on morphological criteria (reviewed by Euler et al., 2014, whereas the zebrafish retina even harbors 17 morphologically distinct subtypes (Figure 1.2) (Connaughton, Graham, and Nelson, 2004). This may be related to the likewise higher number of cone photoreceptor types found in zebrafish: Zebrafish possess red-, green- blue- (Robinson, Schmitt, and Dowling, 1995) and UV- sensitive cone types (Robinson et al., 1993). Although our understanding of bipolar cell function has greatly improved during the last years, also due to recent advances in Ca^{2+} (Dreosti et al., 2009; Baden et al., 2013; Yonehara et al., 2013) and glutamate (Marvin et al., 2013; Borghuis et al., 2013) imaging, it is largely still unknown how the individual morphologically defined bipolar cell subtypes shape the visual signal.

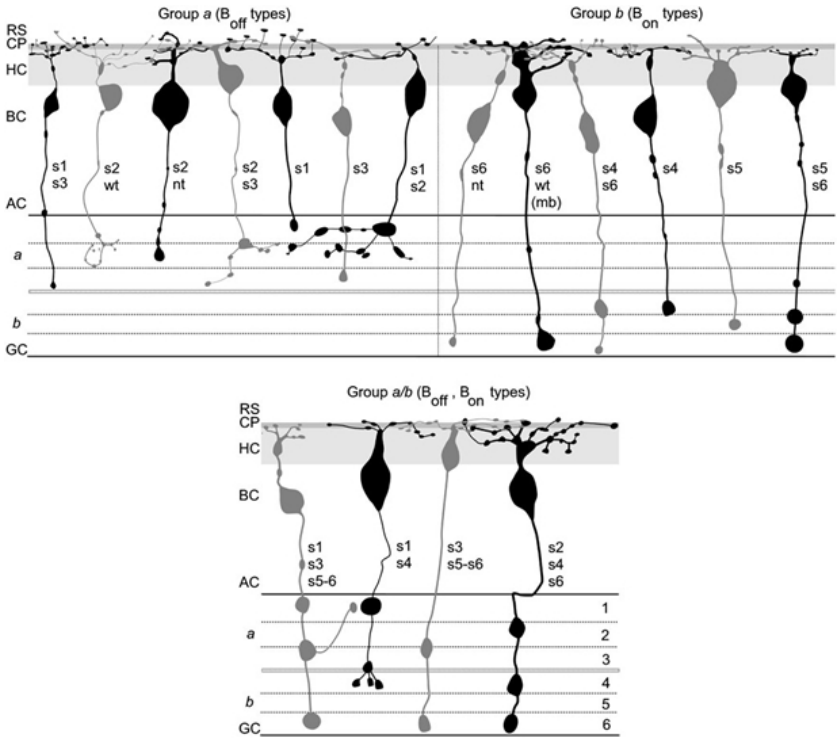

FIGURE 1.2: **Bipolar cell subtypes in the zebrafish retina.** 17 morphologically distinct subtypes have been identified, mainly based on shape and position of the cell body and morphology and projection depth in the inner plexiform layer (IPL). Bipolar cells are grouped into presumptive OFF types stratifying in sublayer a of the IPL, preumptive ON types stratifying in sublayer b of the IPL and presumptive mixed ON/OFF types stratifying in both sublaminae. Letters to the left indicate positions of cell bodies of bipolar and other retinal cell types. AC, amacrine cell; BC, bipolar cell; CP, cone pedicles; GC, retinal ganglion cell; HC, horizontal cell; rs, rod spherules From (Connaughton, Graham, and Nelson, 2004).

1.5 The zebrafish in retina research

Today, the zebrafish is one of the world's most popular model organisms. After being recognized by George Streisinger as a promising vertebrate model, zebrafish have initially gained their popularity in the field of developmental biology. Already early on in zebrafish research, the developmental aspect of zebrafish vision was being investigated (Streisinger et al., 1989). Today, owing to its genetic amenability and other advantages, the zebrafish is becoming increasingly used in retina research and other fields of neurobiology. To allow translation of insights gained from a model organism to other vertebrates such as humans, basic vertebrate features need to be retained, something that is true for many aspects of zebrafish biology. The retina is an anatomical structure highly conserved across most vertebrates, and also in zebrafish (Figure 1.3).

Zebrafish vision develops extraordinarily fast. At 5 days post fertilization (dpf), when zebrafish larvae start vision-driven foraging, their retina

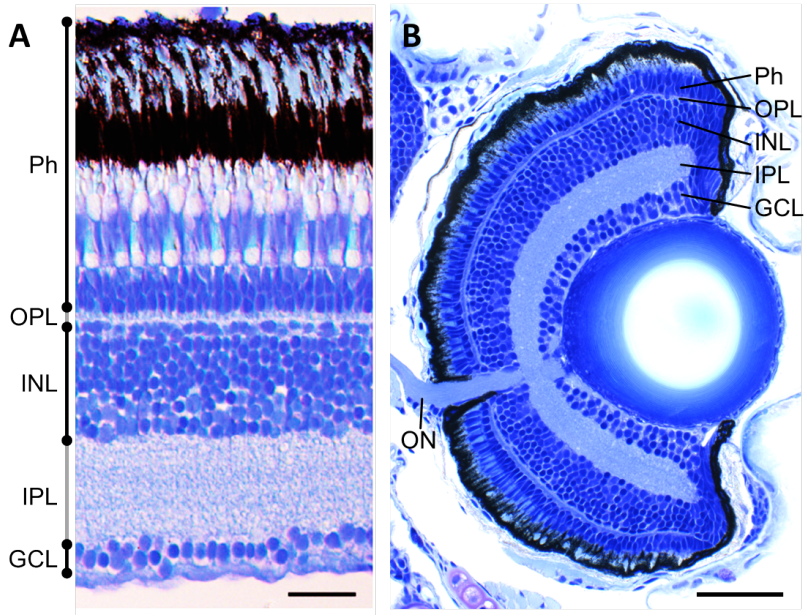

FIGURE 1.3: **Anatomy of the zebrafish retina.** The typical layered structure of the vertebrate retina is apparent both in the adult **(A)** and the larval retina at 5 days post fertilization **(B)**. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ON, optic nerve; OPL, outer plexiform layer; Ph, photoreceptor layer. Scale bars correspond to 25 µm **(A)** and 50 µm **(B)**. **(A)** adapted form (Gramage, Li, and Hitchcock, 2014).

is considered fully functional and basically already exhibits adult morphology (Figure 1.3). Early zebrafish vision relies solely on cones, as rods are integrated into the retinal circuitry days later. Rod signaling is regarded to be functional from around 15 dpf on (Branchek, 1984). However, owing to their diurnal lifestyle in well-lit shallow water, zebrafish have a requirement for well-developed photopic vision throughout life. In this respect, zebrafish vision is more similar to human vision than the one of mice with their nocturnal life style and rod-dominated retina. The zebrafish retina, especially the larval one, is therefore ideally suited to study cone function and photopic vision in general.

Another important feature of the zebrafish model, and a major reason for its popularity, is its amenability to genetic manipulations. Earlier forward genetic approaches have used ENU (Mullins et al., 1994; Solnica-Krezel, Schier, and Driever, 1994; Driever et al., 1996; Mullins et al., 1996) and retroviruses (Gaiano et al., 1996) as mutagens, and many mutant lines useful in vision research were discovered (Malicki et al., 1996; Neuhauss et al., 1999; Brockerhoff et al., 1995; Muto et al., 2005). As complete genomic information of the zebrafish is available (Howe et al., 2013), also reverse genetic approaches can be undertaken. Antisense-based knockdown mediated by morpholinos has been used extensively and recently, the CRISPR/Cas9 technology has been implemented in zebrafish (Hwang et al., 2013), making it now possible to mutate virtually any gene of interest. In addition, Tol2 transposon mediated transgenesis can efficiently be

applied (reviewed by Kawakami, 2007) for labeling and manipulating cells of interest.

The genetic toolkit is complemented by well-established read-outs of retinal function. Electroretinography, frequently used for diagnosis in humans, has been adapted to zebrafish larvae to investigate dysfunctions of the outer retina (Makhankov, Rinner, and Neuhauss, 2004), and investigating the optokinetic response (reviewed by Huang and Neuhauss, 2008) can be used to detect defects such as compromised visual acuity (Haug et al., 2010) and color blindness (Brockerhoff et al., 1997).

1.6 Objectives of the thesis

In this thesis, I have used the zebrafish as model organism to investigate cellular and molecular aspects of retinal function.

Although our knowledge of bipolar cells' functions has significantly increased over the past years, the role of morphologically defined bipolar cell subtypes in retinal processing is still poorly understood. In an attempt to label different subtypes transgenically and make them experimentally amenable in this way, we aimed to identify novel genes specifically expressed in retinal bipolar cells (Chapter 3, page 45), and to isolate regulatory regions of such genes in order to drive transgene expression in bipolar cells, preferably in a subtype-specific manner (Chapter 4, page 55).

Such a transgenic line, which was created by using regulatory sequences of *mglur6b*, exhibits interesting transgene expressing domains besides bipolar cells. Since these expression domains open the possibility of hyperpolarizing responses to glutamate at synapses besides the photoreceptor-ON bipolar cell synapse, we investigate this line in detail in Chapter 5 (Page 73.

Mutations in genes encoding proteins expressed at the photoreceptor ribbon synapse have been linked to several retinal disorders. One such gene encodes the auxiliary $\alpha_2\delta_4$ subunit of voltage-gated Ca^{2+} channels, and its loss causes cone dysfunction in humans. The cone-dominant zebrafish is a promising model to elucidate onset and progression of this retinal disorder. Thus, in Chapter 6 (Page 101), we examine expression and function of $\alpha_2\delta_4$ encoding genes in zebrafish.

Zebrafish and all other extant teleosts are descendants of a ray-finned fish ancestor that underwent whole-genome duplication. Since many genes expressed in the retina have been retained as duplicates after this event, a re-occurring topic in all the studies presented here is the evolution of duplicated genes. Chapter 2 (Page 13) is dedicated to teleost-specific whole genome duplication and its consequences.

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Chapter 2

Whole-Genome Duplication in Teleost Fishes and Its Evolutionary Consequences

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2.1 Abstract

Whole genome duplication (WGD) events have shaped the history of many evolutionary lineages. One such duplication has been implicated in the evolution of teleost fishes, the by far most species-rich vertebrate clade. After initial controversy, evidence is now solid that such event took place in the common ancestor of all extant teleosts. It is termed teleost-specific (TS) WGD. After WGD, duplicate genes have different fates. The most likely outcome is non-functionalization of one duplicate gene due to the lack of selective constraint on preserving both copies of the gene. Mechanisms that act on preservation of duplicates are subfunctionalization (partitioning of ancestral gene functions on the duplicates), neofunctionalization (assigning a novel function to one of the duplicates) and dosage-selection (preserving genes to maintain dosage-balance between interconnected components). Since the frequency of these mechanisms is influenced by the gene's properties, there are over-retained classes of genes, such as highly expressed ones and genes involved in neural function. The consequences of the TS-WGD, especially its impact on the massive radiation of teleosts, have been matter of controversial debate. It is evident that gene duplications are crucial for generating complexity and WGDs provide large amounts of raw material for evolutionary adaptation and innovation. However, it is less clear whether the TS-WGD is directly linked to their evolutionary success and radiation. Recent studies let us conclude that TS-WGD has been important in generating teleost complexity, but that more recent ecological adaptations only marginally related to TS-WGD might have even contributed more to diversification. It is likely, however, that TS-WGD provided teleosts with diversification potential that can become effective much later, such as during phases of environmental change.

2.2 Introduction

During evolution, genes are often subject to duplication events. Duplications can affect single genes, a stretch of several genes, whole chromosomes or even whole genomes. Doubling of whole genomes initially leads to polyploidization (doubling of the whole chromosomal set) and can principally be achieved by nonreduction in meiosis or somatic doubling in mitosis, either in the parental germline or in the early embryo. During evolution, however, polyploidy often does not persist. Duplicated chromosomes accumulate changes until they become too different to pair as quadrivalents during meiosis. Eventually, when disomic inheritance of all chromosomes is restored, a fully diploid organism emerges. This process is called rediploidization. While re-diploidized organisms are no longer polyploid, they still carry signs of the ancestral polyploidization event, such as genes that have been retained as duplicates.

Duplication of a gene results in two daughter genes, termed paralogues (resulting from a duplication event within the genome regardless of the mechanism they arose by). Immediately after duplication, paralogues are identical and functionally redundant. It was realized early on by Susumu Ohno that such redundant genes are attractive candidates to provide the genetic raw material for evolutionary innovation (Ohno, 1970a). By releasing genes from selective constraint in this way, one of the duplicates can be assigned a novel function, a process called neofunctionalization.

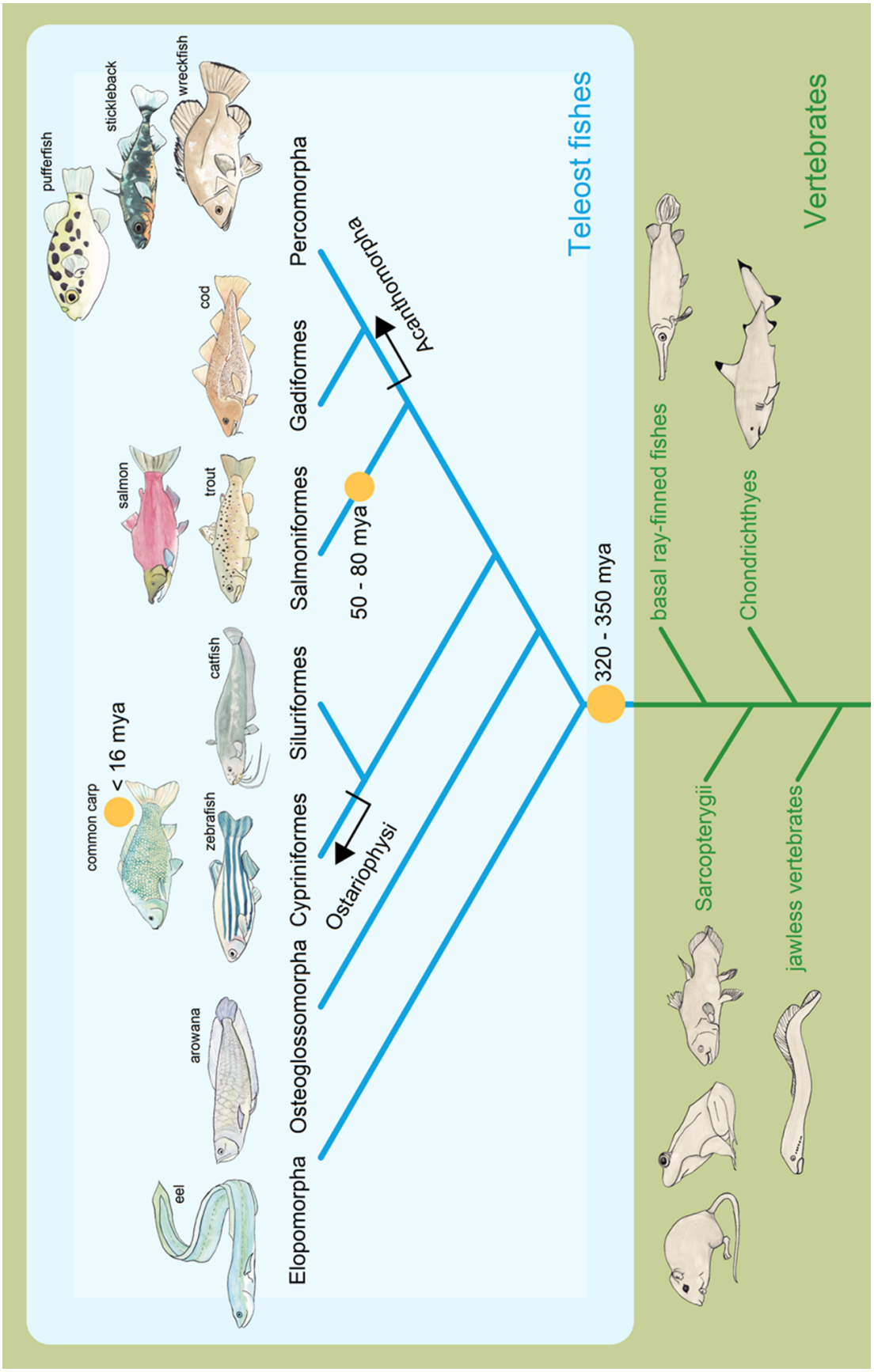
It has been suggested that whole genome duplications are especially important in generating novel genes, since during whole-genome duplication WGD the entire genetic repertoire of an organism is doubled. Although the importance of polyploidization events has initially been realized in plants, it is now clear that also many animals experienced WGDs (Mable, 2004). Even within mammals, which are generally thought not to tolerate polyploidization, a duplicated genome was identified in a rodent (Gallardo et al., 1999; Gallardo et al., 2004). There is now clear evidence that the radiation of vertebrates was preceded by two rounds of WGD, and it has been suggested that these events have contributed to diversification and evolutionary innovations within vertebrates (Canestro et al., 2013).

The subject of this review is to focus on a third round of WGD within vertebrates that occurred at the base of the teleost fish lineage, termed teleost-specific (TS) WGD. Teleosts comprise most extant bony fishes and are the by far most diverse vertebrate group. First, we will summarize evidence for this WGD event. We will then discuss the fate that duplicate genes can undergo, especially in the context of genes that originated in the TS-WGD. Finally, we are asking which evolutionary consequences the TS-WGD had, focusing on its potential contribution to the massive radiation of teleost fishes.

2.3 WGDs have shaped teleost evolution

2.3.1 A WGD took place in the common ancestor of all teleosts

First evidences for TS-WGD emerged from the realization that many tetrapod genes have two orthologues in teleosts (Wittbrodt, Meyer, and Schartl,


Vertebrates
jawless vertebrates
Sarcopterygii
Chondrichthyes
basal ray-finned fishes
320 - 350 mya
Teleost fishes
Elopomorpha
Osteoglossomorpha
Cypriniformes
Siluriformes
Salmoniformes
Gadiformes
Percomorpha
Ostariophysi
50 - 80 mya
Acanthomorpha
< 16 mya
eel
arowana
zebrafish
common carp
catfish
trout
salmon
cod
wreckfish
stickleback
pufferfish

1998; Taylor, Van de Peer, Yves, and Meyer, 2001). However, it was unclear whether these co-orthologues originated in a single WGD event or in a sequence of smaller duplications at the level of whole chromosomes or chromosomal pieces. Several lines of evidence confirm that a WGD indeed took place at the root of the teleost lineage:

Early on, the developmentally important and well-conserved Hox gene clusters sparked interest. After identifying many supernumerous Hox genes to the already known ones from tetrapods (Njolstad et al., 1988; Misof and Wagner, 1996; Aparicio et al., 1997), the systematic evaluations of Hox genes revealed seven Hox clusters in zebrafish (Amores et al., 1998; Prince et al., 1998) as opposed to the four found in tetrapods. Although duplicated Hox genes have also been identified in other teleosts, it was initially not clear whether the increase in the number of Hox clusters is universal for teleosts (Prohaska and Stadler, 2004). Recently, duplicated Hox gene clusters were found in the two most basal extant groups of teleost fishes, the Elopomorpha (including eels and tarpons) (Guo, Gan, and He, 2009; Henkel et al., 2012) and Osteoglossomorpha (including bony tongues and elephantfish) (Chambers et al., 2009). Notably, the eels (European and Japanese eel) are currently the only fishes in which the complete set of the original eight Hox clusters has been observed (Guo, Gan, and He, 2009; Henkel et al., 2012). Since Elopomorpha is the most basal teleost group (Arratia, 1997; Near et al., 2012) this strongly suggests that the ancestor of all living teleosts also possessed eight Hox clusters, consistent with a WGD at the base of teleost evolution (Figure 2.1).

Since the conservation of long stretches of gene order in the entire teleost lineage is an expected outcome of a WGD, the detection of conserved synteny (gene order on chromosomes) of Hox clusters and other genes in a number of teleost fishes was taken as strong evidence for TS-WGD (Amores et al., 1998; Gates et al., 1999; Barbazuk et al., 2000; Taylor, 2003; Hoegg and Meyer, 2007; Sato, Hashiguchi, and Nishida, 2009). Additional support for TS-WGD has been gained by molecular clock analyses. A WGD is expected to result in the divergence of all the resulting paralogues at the same time. Indeed, in two molecular-clock analyses using the Fugu genome, clear peaks in age distribution of paralogous blocks of duplicated genes were observed (Christoffels, 2004; Vandepoele et al., 2004). Although the age estimates of the fish-specific genome duplication are slightly diverging (350 million years ago (mya) (Christoffels, 2004) versus 320 mya (Vandepoele et al., 2004)), both studies place it before the teleost radiation, which is consistent with a genome duplication at the base of ray-finned fishes.

FIGURE 2.1 *(preceding page)*: Simplified phylogeny of teleost fishes. The teleost lineage split from basal ray-finned fishes and started to diverge after a WGD event that took place 320-350mya. Additional WGDs occured at the base of Salmoniformes 50-80 mya and in a closely related ancestor of the common carp 5.6-11.3 mya. For the sake of clarity, only a selection of teleost taxa is presented. Orange circles depict WGD events within teleost evolution. WGD events outside the teleosts are not shown. Mya, million years ago.

Finally, whole-genome sequencing of a number of fish genomes provided conclusive evidence for at least one WGD in the whole teleost lineage (Aparicio et al., 2002; Jaillon et al., 2004; Kasahara et al., 2007; Howe et al., 2013; Schartl et al., 2013). In summary, owing to convincing evidence on many levels, it is now widely accepted that the TS-WGD took place.

2.3.2 Additional lineage-specific WGDs occurred in salmonids and some cyprinids

Additionally to the WGD at the base of teleost evolution, more recent genome duplications have shaped fish evolution. WGD events are well established for both salmonids and cyprinids. The ancestor of all extant salmonids underwent a tetraploidization event (Johnson, K. R., Wright, J. E. Jr., and May, B., 1987), according to most recent estimations probably between 80 and 50 mya (Alexandrou et al., 2013). From the 1970ies on (Ohno, 1970b), it has been suspected that the salmonid specific genome duplication event, which preceded the origin of migratory behavior, provided the genetic basis for this evolutionary innovation (Alexandrou et al., 2013).

Within Cyprinidae, the common carp and the goldfish have been suggested to be tetraploid (Ohno et al., 1967). Analysis of microsatellite loci (David, 2003) and comparing the linkage map of the common carp to the zebrafish genome (Zhang et al., 2013) provided strong evidence for the duplication event in the common carp. Goldfish and the common carp are closely related and likely share the same tetraploid ancestor which underwent a genome duplication an estimated 5.6 – 11.3 mya (Wang et al., 2012). Additional polyploidization events within the Cyprinidae have been described in some loaches (Cobitidae) (Ferris and Whitt, 1977a) and in suckers (Catostomidae) (Uyeno and Smith, 1972). Once more fish species become subject to genomic analysis, we will likely see many more additional examples of WGDs in teleost sublineages.

2.3.3 The evolution of chromosome numbers after teleost WGDs

WGD initially leads to doubling of the chromosomal set. However, it is well known that chromosomes behave dynamically during evolution and undergo rearrangements, such as centric fusions by Robertsonian Translocation. This mechanism leads to two chromosomes being fused at their centromeres, resulting in a reduction of chromosome number. Approaches to infer the ancestral teleost prior to TS-WGD have consistently predicted a haploid chromosome number of 12 to 13 (Postlethwait et al., 2000; Jaillon et al., 2004; Kohn et al., 2006; Kasahara et al., 2007). Accordingly, TS-WGD resulted in a post-duplication ancestor with 24 or 26 chromosomes. More than 50% of all extant teleosts with data in the genome size database `http://www.genomesize.com` have indeed 24 or 25 chromosomes (Naruse et al., 2004), presumably representing the ancestral condition. Thus, the number of chromosomes remained nearly unchanged during evolution of most extant species. However, whereas the number of chromosomes remained fairly constant, the comparison of different teleost genomes to that of humans revealed a higher rate of chromosomal rearrangements other than fusions (Kasahara et al., 2007). After the recent WGD in the ancestor of common carp and goldfish, chromosome numbers

have also not been reduced. Both species have 50 chromosomes, twice as many as other Cyprinidae (Ohno et al., 1967).

Conserving chromosome numbers after WGD is not essential, as chromosome numbers of salmonids illustrate. Because the stem salmonid underwent WGD, unchanged chromosome numbers would result in extant salmonids with around 50 chromosomes, a number twice that of their closest relatives. In contrast, although cells of salmonid fishes consistently have double the DNA content (Gregory et al., 2007) and chromosome arms (Phillips et al., 2009) as compared to their closest relatives, their chromosome numbers vary extensively between 26 and 51. Most of the species have a lower chromosome number than the original number after duplication (Supplementary Figure 2.S1. Therefore, chromosome fusions must have played a major role in shaping salmonid karyotypes, as proposed by Hartley (Hartley, 1986). Different modes of chromosome evolution appear to have acted in the evolution of different salmonid sublineages, leading to the diverse chromosome numbers observed (reviewed by Phillips and Rab, 2001).

2.4 The fates of duplicated genes after WGD

After WGD, all duplicated genes should be relieved from selective pressure and therefore would be expected to vanish over time. However, the fate of duplicated genes is more complicated and much more interesting (Figure 2.2).

WGD derived duplicate gene pairs can undergo different fates: One of the duplicates may be lost (non-functionalization), both duplicates may be retained basically unchanged, both duplicates may acquire changes so that the function of the ancestral gene is divided among the duplicates (a process called subfunctionalization), and finally one of the duplicate genes may acquire a new function (neofunctionalization). For clarity, we will describe the different scenarios as individual processes. But we like to stress that the categories are simplified, and that multiple scenarios may affect the evolution of individual genes. Different mechanisms can act successively to shape different phases of gene evolution. Furthermore, two or more mechanisms may act on the same duplicate gene pair simultaneously.

2.4.1 Non-functionalization

Immediately after WGD, the daughter genes of each ancestral gene are identical, and their functions are redundant. This suggests that selective constraint of maintaining both of them is low and that one of them is therefore free to disappear due to genetic drift. A classical model, first formulated by Ohno (Ohno, 1970a) predicts that loss of one paralogue is the most common outcome of duplicate gene evolution. This assumption is based on the simple fact that deleterious mutations are much more likely to occur than beneficial ones. Thus, one of the duplicates is expected to accumulate deleterious mutations, eventually leading to its silencing. Indeed, experimental studies have confirmed that non-functionalization is the most common scenario of duplicate gene evolution (Jaillon et al., 2004; Woods et al., 2005; Brunet et al., 2006). Estimates have suggested that as few as 1-5% of duplicate genes have been retained in pufferfish (Aparicio et al., 2002;

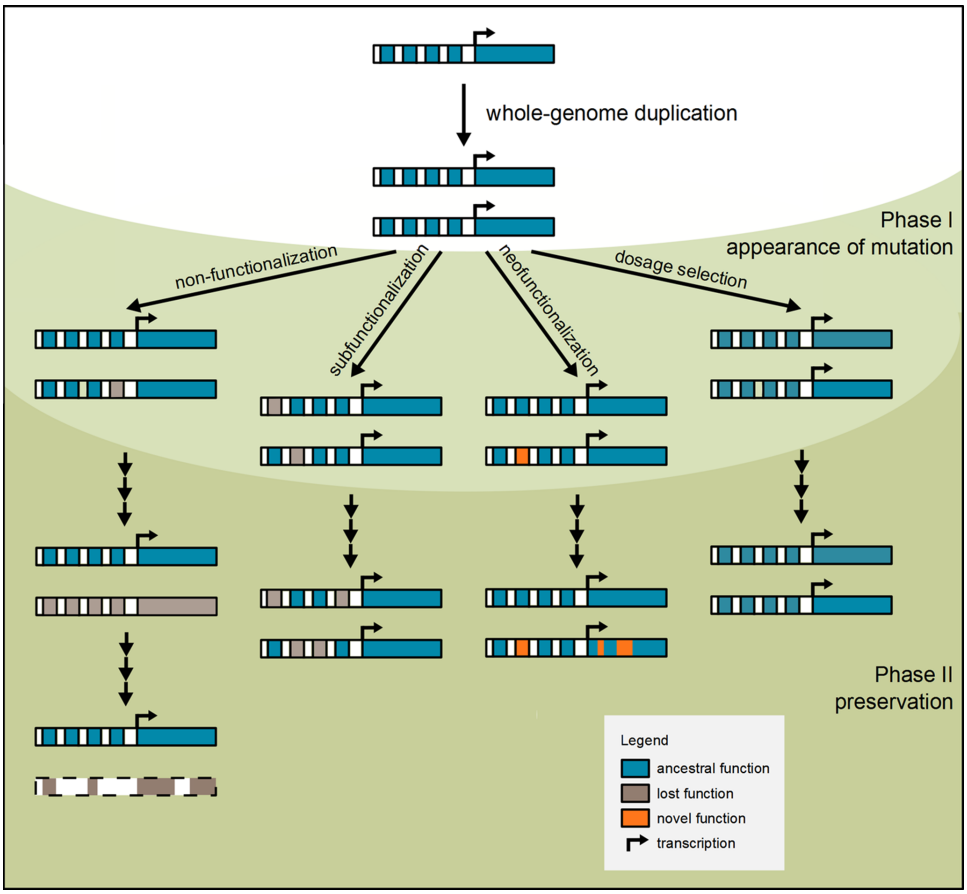


FIGURE 2.2: Fates of duplicated genes after WGD. A WGD event results in the formation of two identical duplicates of every gene. Duplicate genes can undergo different fates. Non-functionalization: Deleterious mutations occur in one of the duplicates, eventually leading to loss of expression (pseudogenization). Mutations continue to accumulate until the structural features of the gene have disappeared completely. Subfunctionalization: Complementary degenerative mutations in paralogous genes lead to preservation of both duplicates. Neofunctionalization: One of the genes acquires a novel function. Dosage selection: Dosage-sensitive genes remain basically unchanged after WGD. Although the initial mutations are depicted in regulatory regions, also changes in coding sequence can lead to the different scenarios. For sake of simplicity, introns were omitted, and regulatory regions are depicted only 5′ of the transcription start site.

Jaillon et al., 2004). This is roughly in agreement with the first genome-wide, comparative analysis of five different fish species by Kassahn et al. (Kassahn et al., 2009) These authors found that in all five species examined, 3-4% of the genes show strong evidence for having originated in the ancient TS-WGD. Due to the design of the study, this number is probably underestimating the real abundance of gene retention after TS-WGD, and can be considered a minimum estimate, as pointed out by the authors themselves. The study also found that there is no difference in the percentage of duplicate gene retention between the five species analyzed. By looking at gene families, other studies have come to the conclusion that up to 20% of TS-WGD-duplicate genes may have been retained in zebrafish (Postlethwait et al., 2000; Postlethwait et al., 2004; Woods et al., 2005), which can be considered the maximum estimate of gene retention rate after the TS-WGD. Gene retention rates after the more recent WGD events in Salmonids and the lineage leading to common carp are much higher: Probably more than 50% of all genes are still present in duplicates in the common carp and salmonids (Ferris and Whitt, 1977b; Allendorf, 1978).

Although non-functionalization is very frequent, Force et al., (1999) noted that the fraction of genes preserved after genome duplication events is higher than predicted by Ohno's classical model (1970). In other words, the probability of a mutation being beneficial versus it being deleterious is too low to explain the number of duplicates observed after WGD events. Force et al. (Force et al., 1999) therefore proposed a seminal model of subfunctionalization with their Duplication-Degeneration-Complementation (DDC) model.

2.4.2 Subfunctionalization by DDC

Genes usually have more than only one function. These functions can be represented by expression in different cell types or developmental stages. Different expression domains are regulated by transcription factors binding to distinct elements in regulatory regions of a gene.

The Duplication-Degeneration Complementation model (Force et al., 1999) proposes that duplicates can be conserved by complementary degenerative mutations in such regulatory regions. The degenerative mutations are neutral, because one gene still performs the ancestral function that was lost in the other one. By this mechanism, functions of a gene can be subdivided between the daughter genes, which – together – continue to perform the functions of their ancestral pre-duplication gene. After complementary loss of subfunctions, both genes will be fixed in the genome, because loss of either of them will disrupt the essential ancestral gene function. It is important to point out that this process can take place in the absence of selection. Genetic drift leading to complementary loss of subfunctions is sufficient to explain the DDC mechanism. A mathematical model provided by Force et al. demonstrates that the higher the number of subfunctions of a gene pair, the higher the probability of fixation by the DDC model, and the lower the probability of non-functionalization (Force et al., 1999)). Furthermore, the model predicts that the fate decision between non- and subfunctionalization is determined quickly on an evolutionary time scale (within a few million years). Taken together, the model suggests that the DDC mechanism has had a significant contribution to the proportion of retained duplicates after WGD that we can observe today. Indeed, numerous cases of TS-WGD

duplicate gene pairs evolving by DDC have been reported (for example, see McClintock et al., 2001; Jovelin et al., 2007; Kassahn et al., 2009; Renninger, Gesemann, and Neuhauss, 2011; von Niederhäusern, Valentin et al., 2013).

One demonstrative example of subfunctionalization is shown by the study of cellular retinaldehyde-binding proteins (CRALBP) in zebrafish (Fleisch et al., 2008). During light perception in photoreceptors, visual pigment absorbs a photon, leading to isomerization of its visual chromophore (11-cis-retinal). The visual pigment is replenished in two separate visual cycles, the canonical cycle located in the retinal pigment epithelium (RPE) and the non-canonical cycle in Müller glia cells (Fleisch and Neuhauss, 2010). The visual chromphore needs to be chaperoned by CRALBP, hence tetrapods express this protein in both RPE and Müller glia cells. In zebrafish there are two CRALBP paralogues, one expressed in Müller glia cells and one in the RPE (Collery et al., 2008; Fleisch et al., 2008). Functional analyses showed that they serve different functions in vision. Hence the ancestral function and expression domain is split up between two paralogues, who together make up the function of the presumptive ancestral gene duplicated at glswgd. Clear examples of subfunctionalization by changes on the amino acid level in animals are rare, but one exemplary case of Proopiomeloncortins in pufferfish duplicated by TS-WGD and subfunctionalized on the amino acid level has been documented (de Souza, Flavio S. J. et al., 2005). Some examples of subfunctionalization on the level of coding sequence after WGD have also been found in plant genomes (Cusack and Wolfe, 2007).

2.4.3 Subfunctionalization by escape from adaptive conflict (EAC)

Besides subfunctionalization by the DDC mechanism, an additional mode of subfunctionalization was first proposed by Hughes, 1994 and has been later termed escape from adaptive conflict (EAC) by DesMarais and Rausher, (2008). In this model, two duplicate genes evolve not solely by genetic drift, but when adaptive evolution is driving changes in both paralogues, leading to their divergence (DesMarais and Rausher, 2008). In this way, better adaptation of a different subfunction in each gene can be achieved. This scenario is expected to occur in cases when two subfunctions of a gene cannot be improved simultaneously, because optimization of one subfunction would negatively interfere with the other one. Duplication solves this conflict and one paralogue is free to acquire adaptive mutations to optimize one subfunction without compromising the performance of the other one, whereas the second paralogue can optimize another subfunction. Whether this model is able to explain a substantial fraction of retained paralogues depends on two factors: The abundance of multifunctional genes and the abundance of situation where optimization of one function impairs another one (Innan and Kondrashov, 2010). Physical modeling of amino acid chains suggests that EAC preferentially takes place under moderate selective pressure and therefore likely in genes that are not essential for survival, but that can substantially improve fitness if optimized (Sikosek, Chan, and Bornberg-Bauer, 2012). So far, duplicate gene evolution by EAC has been mainly a theoretical model and only few cases indicating EAC have been documented (DesMarais and Rausher, 2008; Deng et al., 2010; Huang et

al., 2012). One reason for the scarcity of examples is related to the difficulty to ascertain whether the criteria for EAC are fulfilled, in particular whether functions were improved compared to the ancestral gene and whether this improvement was really constrained before duplication (Barkman and Zhang, 2009).

2.4.4 Neofunctionalization

Besides non- and subfunctionalization, duplicate genes can also acquire novel functions. This is the classical model of neofunctionalization of one paralogue, again first formulated by Ohno, (1970a). It is also referred to as "mutation during non-functionality" (MDN) model (Hughes, 1994; Conant and Wolfe, 2008). Due to the lack of selective constraint on maintaining both duplicates, one of them is free to acquire mutations conferring a new function. As discussed above, beneficial mutations occur only at a low rate. Therefore, this scenario is expected to be encountered less frequently than non- or subfunctionalization. Indeed, fewer instances of neofunctionalization have been confirmed. The technical difficulty to identify cases of neofunctionalization likely greatly contributes to the apparent scarcity of bona fide examples (Conant and Wolfe, 2008). Duplicate genes with divergent functions, one of which is new, might be gene pairs that underwent neofunctionalization. Instances of neofunctionalization that have been reported are frequently gain of novel expression domains, and therefore probably neofunctionalization by alterations in their regulatory regions (Kassahn et al., 2009), while changes in the coding sequence of a genes giving rise to a new function are rarer (Braasch, Salzburger, and Meyer, 2006; Douard et al., 2008).

One illustrative example of neofunctionalization in both regulatory and protein coding sequences of a TS-WGD duplicate gene pair is the co-option of a voltage-gated sodium channel to contribute to the origin and function of electric organs (Zakon et al., 2006; Arnegard et al., 2010). Both African mormyroid and South American gymnotiform fishes possess organs to electrically generate communication signals. Interestingly, although the electric organs of those two groups are very similar (for example, they are both derived from skeletal muscle), they evolved independently (Alves-Gomes, 1999). The electric organ was therefore invented twice by convergent evolution. The function of electric organs highly depends on modified voltage-gated sodium channels that are needed to discharge electrocytes, the cells of the electric organs suitable to produce the communication signal. Teleost fishes possess *scn4aa* and *scn4ab*, two products of TS-WGD coding for alpha-subunits of voltage-gated sodium channels. In non-electrogenic fishes, both paralogues are expressed in skeletal muscle. But in all members of both groups of electrogenic fishes, expression of *scn4aa* was found to be lost from muscle and gained in the electric organ. The fact that this switch in expression is found even in the most basal electrogenic fishes in both groups suggests that *scn4aa* not only acts in, but also supported the formation of the electric organs (Arnegard et al., 2010). Furthermore, the authors found that selective pressure acted specifically on the protein-coding sequence of the *scn4aa* paralogue during phases when the electric organ was evolving, whereas selective pressure on *scn4ab* remained constantly low. Strikingly, selective forces showed to be particularly high

in functionally important regions of the proteins, for example in extracellular loops that are thought to have an impact on the duration of electric organ discharge and therefore on the properties of the communication signal. These specific changes occurred in parallel in types of gymnotiform and mormyroid electric fishes that generate pulsed electric signals as opposed to the more uniform signals of their relatives. In summary, the innovation of electric organs in two distant related groups of weak electric fishes highlights two interesting aspects: Firstly, it demonstrates that genes which arose in genome duplications can acquire new functions, leading to the acquisition of evolution of new evolutionary traits, even complex ones such as new organs. Secondly, it illustrates an example of pre-adaptation and co-option: The duplicated sodium channel existed in the genome of fishes and probably acted in muscle activity for around 100 million years (Arnegard et al., 2010) until, within an evolutionary short period of time, it was co-opted twice to function in the electric organ.

2.4.5 Gene dosage effects

A final mechanism of duplicate gene retention worth discussing here is retention due to dosage effects. Directly after WGD, all chromosomes and genes are doubled in every cell, and therefore it can be assumed that the duplicate gene pairs are all expressed at a higher level than the corresponding ancestral gene. Since this is true for every gene, relative gene dosage is not disrupted by WGD. Maintaining gene dosage balance seems to be crucial for some genes, and loss of dosage-sensitive genes after WGD can be detrimental. Degenerative mutations of such genes disrupt balanced expression of genes interconnected in networks. Because relative gene dosages of such genes are important, reducing gene dosage by deleterious mutations in one paralogue can lead to negative developmental or physiological consequences. Genes where dosage is believed to be especially important are ribosomal genes, genes coding for proteins with a high number of interactions, and genes encoding proteins functioning in signaling pathways and networks. Requirement for gene dosage maintenance can lead to scenarios in which all genes of a network or pathway remain duplicated, and it has been suggested that retention of duplicate members in whole networks, can have broad evolutionary implications (Conant and Wolfe, 2007).

Support for this Gene Balance Hypothesis comes from comparing trends in gene retention between single-gene duplications and WGDs. Relative gene dosages are after a WGD initially not changed, while single-gene duplications instantaneously disrupt gene dosage balance and should therefore be selected against in dosage-sensitive systems. Indeed, it has been shown in vertebrates and plants that highly interconnected genes, such as genes involved in transcription and signaling cascades, and genes coding for proteins with more than average protein-protein interactions are over-retained after WGDs, but not after small-scale duplications (Blomme et al., 2006; Freeling, 2008; Hufton et al., 2009).

Hufton et al. even propose that the gene balance hypothesis better explains gene duplication retention in vertebrate genomes than the DDC model (2009). In their study of phylogenetically conserved noncoding sequences, they showed that genes retained after WGD are rather marked by many protein interaction sites than by many conserved noncoding elements, as

the DDC model would predict (Hufton et al., 2009). Additionally, it is plausible that an increased dose of some genes is beneficial even if they are not highly interactive. In such a case, both duplicates will be preferentially retained in the genome as well. Examples for genes that are required in high doses and that are therefore prone to be maintained as duplicates by positive selection are histones and ribosomal proteins (Sugino and Innan, 2006). However, both histones and ribosomal proteins are also dependent on the abundance of their interaction partners (other histones, other ribosomal proteins and ribosomal RNA), and might therefore be retained by both the benefits of an increased dose and the need to keep dosage balance.

As noted at the beginning of the section, the different mechanisms leading to different fates cannot be regarded as isolated processes since they can act together, resulting in complex evolutionary dynamics of duplicate genes. On top of that, outcomes of duplicate gene evolution are also affected by other interesting evolutionary mechanisms such as gene conversion.

2.4.6 Concerted evolution by gene conversion

Gene conversion has been described as nonreciprocal exchange of DNA fragments between homologous sequences within a genome. Gene conversion can also be regarded as a copy-and-paste event by which a gene fragment is replaced by a homologous sequence. For further reading on the mechanistic basis of gene conversion we suggest a review by Chen et al., (2007). When gene conversion is active between genes at a sufficiently high rate, those genes do not evolve independently anymore, but in a fashion called concerted evolution. The principal effect of concerted evolution is that affected genes remain more similar to each other than would be expected considering only divergent evolution without gene conversion.

Gene conversion is dependent on homology and sufficient sequence similarity (Ahn et al., 1988; Elliott et al., 1998), and can therefore also be expected to be active between paralogues arising through WGDs. Several effects of gene conversion on duplicated genes have been suggested (reviewed by Innan, 2009): First, gene conversion is expected to make nonfunctionalization less likely, since deleterious mutations in one duplicate can be removed by "pasting" the corresponding sequence of its intact paralogue. Second, gene conversion can contribute to neofunctionalization, since beneficial mutations can be shared and also, novel combinations of allelic sequences can be created. In a theoretical model, Teshina and Innan have explored another interesting effect of gene conversion by which it counteracts neofunctionalization (Teshima and Innan, 2008). A DNA sequence conferring a novel function can be converted back to the ancestral sequence by gene conversion. Therefore, in genes undergoing conversion, neofunctionalization can only occur under strong selection. A second consequence suggested by this study is that gene conversion prevents complete fixation of a novel gene. As a result, fixation of neofunctionalization can only take place subsequently to mechanisms that terminate gene conversion, i.e. progressive sequence divergence or events of immediate large impact such as transposon insertions. Clear examples of gene conversion are documented in plants, fungi and animals (for example, see Semple and Wolfe, 1999; Drouin, 2002; Rozen et al., 2003; Mondragon-Palomino and

Gaut, 2005). It has also been shown that gene conversion can principally be active after WGD by studies in yeast (Wolfe and Shields, 1997; Kellis, Birren, and Lander, 2004). However, the extent to which gene conversion contributes to duplicate gene conversion, in particular after WGDs, is still unclear due to difficulties in detecting gene conversion with current methods (Mansai and Innan, 2010).

Almost all gene conversion events discovered in teleosts have affected paralogues that do not stem from TS-WGD but rather from more recent duplications (mostly tandem duplications) in teleost sublineages (Bargelloni et al., 1999; McGuigan, Phillips, and Postlethwait, 2004; Noonan et al., 2004; Gerrard and Meyer, 2007; Yu et al., 2007; Windsor and Owens, 2009; Weadick and Chang, 2012). Only one instance of gene conversion between paralogues generated by TS-WGD, namely rainbow trout *sox9α2* and *sox9* (Alfaqih et al., 2009) has so far been documented. However, the scarcity of examples does not necessarily imply that gene conversion was infrequent or unimportant after TS-WGD. Since gene conversion depends on sufficient sequence similarity between paralogues, it is expected to be most common directly following WGD events and becoming less frequent over the course of time. Therefore, many gene conversion events acting on paralogues duplicated by TS-WGD can be expected to be ancient and quite hard to detect.

2.4.7 Coding and noncoding regions in duplicate gene evolution

Most of the mechanisms we have discussed can either be achieved by mutations in coding- or noncoding regions. In the case of subfunctionalization, gene expression of duplicate genes can be divided between tissues by reciprocal loss of cis-regulatory elements, as Force et al. initially postulated (1999). However, distribution of an ancestral function onto two paralogous genes can in principal also be achieved by reciprocal inactivation of functional domains. Similarly, a new function can be gained through the addition of another regulatory element resulting in a new expression domain, or through imposing a new function via changes within the coding sequence.

It is still lively debated whether changes in coding- or non-coding sequences are more relevant to the evolution of genes and new traits (Carroll, 2000; Hoekstra and Coyne, 2007; Wray, 2007; Lynch and Wagner, 2008; Wittkopp and Kalay, 2012). One of the difficulties is that it is straightforward to detect changes in coding regions, while cis-regulatory elements are small, interspersed with non-relevant sequences, often far away from the regulated gene, and less strictly conserved in sequence. Furthermore, their position can be changed or they can be inverted without functional consequences. Therefore meaningful changes in non-coding sequences are much harder to identify. Hence it is no surprise that studies having directly identified cis-regulatory sequence changes as the source of divergent duplicate gene expression are rare. However, the consequences of cis-regulatory mutations can easily be identified by expression analyses, such as (quantitative) reverse PCR and RNA in-situ hybridization. In fact, changes in expression patterns have often been interpreted by authors as alterations in cis-regulatory regions. This may often be the appropriate conclusion, however the methods used might not always detect alternatively spliced variants of a paralogue which might be prevalent in a certain tissue, and

mRNA abundances can also be altered through changes in mRNA stability (Hoekstra and Coyne, 2007).

Comparative genome wide analyses of retained duplicates after the TS-WGD from five species (Kassahn et al., 2009) showed that expression patterns often diverge between paralogues: 87% of duplicate gene pairs showed distinct expression patterns (indicative of neo- and subfunctionalization events) in at least one developmental stage examined. This number represents only a rough estimate since it might underestimate the actual number of divergent genes. Some paralogues might be differentially expressed in developmental stages not examined. This is especially relevant since adult stages are often not included in expression analyses, and the same survey showed that expression patterns of duplicate gene pairs get more distinct during development.

This study suggests that neofunctionalization through changes in regulatory regions might be more abundant than predicted by the classical model of neofunctionalization by Ohno, (1970a). This discrepancy can be alleviated if the Duplication Degeneration Innovation (DDI) model proposed by Jiménez-Delgado and coauthors is taken into account (Jimenez-Delgado, Pascual-Anaya, and Garcia-Fernandez, 2009). In the DDI model, sub- and neofunctionalization act together on regulatory elements to achieve evolutionary innovation. After duplication and during degeneration, conserved non-coding elements (CNEs) become non-functional, but retain their structural enhancer properties. Therefore, expression in a new spatial and/ or temporal manner can be achieved even by only subtle mutations in those degenerate CNEs, making neofunctionalization more likely to occur.

When contemplating evolutionary divergence of coding regions, the first events that come to mind are amino acid substitutions caused by non-synonymous point mutations. While this mechanism has received considerable attention for decades, there is accumulating evidence that other mechanisms, which have been started to be investigated more recently, are also significantly contributing to structural and functional divergence of proteins after genome duplications. Divergence of coding regions can be achieved by a number of mechanisms other than point mutations. These include insertions/deletions (indels), exon gain/loss, exonization/ pseudoexonization, exon suffling and divergence of alternative splicing. Divergence in splicing and indels have been suggested to contribute substantially to evolution after WGD.

Divergence of splicing events can principally play a role after polyploidization as shown in a study in plants (Brassicaceae) (Zhou, Moshgabadi, and Adams, 2011). In this study natural and resynthesized tetraploid species were compared to a closely related diploid species in terms of splicing patterns of paralogous genes. A substantial number (>20%) of paralogous gene pairs were found to have diverged in splicing events. The resynthesized tetraploids showed that those changes occur fast: Already after 5 generations, more than 20% of duplicate gene pairs showed divergent splicing patterns. The study also showed that the most common change in splicing is loss of one parental splicing event in a duplicate gene. Whether such a mechanism is equally prevalent in teleost evolution is not known, but provides an attractive alternative mechanism to explain duplicate gene retention due to partition of alternative splicing between paralogues.

Indels have also been shown to very frequently contribute to divergent gene evolution after the TS-WGD. Both members of a paralogous gene pair have experienced significantly more insertion and deletion events than genes not retained as duplicates. These indels mostly occurred shortly after the duplication event and are predicted to affect protein structure more than amino acid substitutions (Guo, Zou, and Wagner, 2012). This and other reports (Brunet et al., 2006; Jiang and Blouin, 2007; Tian et al., 2008; Chen et al., 2009) have led to the idea that Indels have at least as much impact on duplicate gene evolution as nucleotide substitutions do.

A number of studies were conducted aiming to address evolution in both coding and noncoding regions of TS-WGD duplicates. In some cases, both noncoding and coding sequences of certain paralogues were found to undergo divergent evolution. In particular, such scenarios were shown for Proopiomelanocortins, prohormones mostly expressed in the pituitary gland (de Souza, Flavio S. J. et al., 2005), and Follistatins, TGF-β binding proteins involved in muscle development (Macqueen and Johnston, 2008). Other studies exclusively identified divergent evolution in noncoding regions, while the coding sequence or gene function remained highly conserved. This was true for a duplicated gene pair of the argonaute (Ago) family, encoding AGO proteins important for small RNA mediated gene silencing (McFarlane et al., 2011), and for IGFBP-2 genes, binding and regulating actions of Insulin-like growth factor (Zhou, Moshgabadi, and Adams, 2011). None of these studies reported identical expression patterns of TS-WGD duplicates, emphasizing that changes in regulatory elements are very common after WGD.

2.4.8 Over-retained duplicates after WGDs

Having discussed the mechanisms for duplicate gene retention, an obvious follow-up question is if the retained genes are distributed equally among gene categories. Strong evidence, mainly obtained in plants, yeast and unicellular eukaryotes has been collected that duplicate genes with certain properties are over-retained after WGD events (for example, see Seoighe and Wolfe, 1999; Papp, Pal, and Hurst, 2003; Maere et al., 2005; Aury et al., 2006.

In agreement with the dosage-balance hypothesis, over-retained duplicate genes often encode proteins with more than average protein-protein interactions and proteins that function in complexes (Hakes et al., 2007). Correspondingly functional categories that have been over-retained include ribosomal proteins, protein kinases and transcription factors. Two recent studies have shown that gene expression is a factor highly correlated with duplicate gene retention. Gout et al. investigated the relation between gene expression and duplicate gene retention on a genome-wide basis in the unicellular Paramecium tetraurelia (Gout, Kahn, and Duret, 2010). The evolutionary lineage leading to *P. tetraurelia* is marked by three rounds of WGDs. There was a strong positive relationship between expression level and duplicate gene retention. Similarly, Chain et al. found that expression level is the factor correlating most strongly with duplicate gene retention in tetraploid *Xenopus laevis*, again suggesting dosage sensitivity of retained duplicates (Chain, Frederic J. J., Dushoff, and Evans, 2011). Evenness of expression was the second strongest factor positively associated with duplicate

gene retention. "Evenness" of expression means activation in many tissues and therefore might be linked to pleiotropy (multifunctionality) and complexity of regulatory sequences, features that increase the chance of sub- and neo functionalization.

Another observation made in both *P. tetraurela* and *X. laevis* was that genes which have been evolving slowly before a genome duplication are more likely to be retained (Gout, Kahn, and Duret, 2010; Chain, Frederic J. J., Dushoff, and Evans, 2011). Consistent with this finding, Semon and Wolfe have previously argued that slowly evolving genes may tend to persist because they give sub- or neofunctionalization more time to take place before deleterious mutations occur (Semon and Wolfe, 2008). An observation that points to the same direction is that in pufferfish, well conserved genes with close homologues already present in invertebrates are overrepresented among duplicates (Kassahn et al., 2009).

By assigning functions to duplicate and non-duplicate genes of five teleost species, it was shown that a number of functional categories is strongly enriched among paralogues derived from TS-WGD (Kassahn et al., 2009). The categories most enriched are related to ion channel and transporter activity. Ion transport needs to be tightly regulated in any cell. However, neurons are the cells that most strongly rely on a repertoire of diverse ion channels and transporters. Consistent with this genome-wide analysis, also studies of protein families in zebrafish have shown that genes involved in neuronal function have often retained both paralogues (Gesemann et al., 2010; Di Donato et al., 2013; Haug et al., 2013; Kastenhuber et al., 2013).

2.5 Consequences of TS-WGD for fish evolution

Teleost fishes represent the by far most diverse vertebrate clade, constituting more than 32,000 species of an estimated total number of 64,000 vertebrate species (Froese and Pauly, 2013). Teleost fishes populate a wide range of oceanic and freshwater habitats all over our planet, ranging from arctic to tropic regions. Without doubt, teleosts are an evolutionary highly successful group.

WGDs are found at the base of some other diverse taxa. The vertebrate stem is marked by two rounds of WGD (Dehal and Boore, 2005; Putnam et al., 2008; Kuraku and Meyer, 2009). Similarly, ancient WGDs have been documented in flowering plants (Jaillon et al., 2007; Tang et al., 2008). Additionally in flowering plants, more recent WGDs at the base of diverse subgroups have been reported (reviewed by Soltis et al., 2009). These and similar observations made in Fungi and unicellular eukaryotes (Aury et al., 2006; Scannell, Butler, and Wolfe, 2007) have led to the idea that there is a causal correlation between WGD, evolutionary success and radiation.

Here, we are going to discuss the impact of TS-WGD on teleost evolution, focusing on its role in their radiation. First, we will briefly summarize the mechanisms by which WGDs are thought to impose selective advantage and facilitate speciation. For an extensive review on this matter, the reader is referred to Van de Peer, Y, Maere, and Meyer, 2009. Then, we will discuss the current and controversial state of evidence on teleost radiation driven by TS-WGD.

2.5.1 Mechanisms by which WGDs can contribute to evolutionary success and radiation

Although polyploidization most often leads to an evolutionary dead end, it seems that polyploid organisms sometimes have advantages over their diploid relatives. In particular, some polyploids have been suggested to be more robust to changing environments, therefore having reduced risk of extinction (Fawcett, Maere, and Van de Peer, Yves, 2009). Rapid genomic and epigenetic changes taking place after WGD (Osborn et al., 2003) probably enable polyploids to adapt faster than diploids. Furthermore, polyploids have been suggested to have increased mutational robustness, meaning that redundant genes copies can transiently mask the effect of deleterious mutations in their paralogue (Otto and Whitton, 2000).

WGDs have also been suggested to directly facilitate speciation by reciprocal gene loss, where different paralogues are lost in different populations, ultimately leading to genetic isolation and speciation of these populations (Scannell et al., 2006). There is indeed evidence for reciprocal gene loss in teleost lineages. It was found that 8% of gene loci of *Tetraodon nigroviridis* (green spotted puffer) and zebrafish underwent reciprocal gene loss (Scannell, Butler, and Wolfe, 2007). Similar to reciprocal gene loss, also subfunctionalization has the potential to lead to genetic isolation of populations (Lynch and Force, 2000; Postlethwait et al., 2004; Volff, 2005).

Also evolutionary innovations made possible by WGD provide a path to evolutionary success. Gene duplication of any kind is a crucial generator of raw material for evolutionary innovation. As discussed before, however, WGDs uniquely enable duplication of dosage sensitive genes. Such genes include regulatory genes, thought to eminently contribute to the emergence of evolutionary innovations. Regulatory gene have also been over-retained in fishes (Blomme et al., 2006; Brunet et al., 2006). Additionally, dosage sensitivity is expected to result in the retention of whole transcriptional networks that can as a whole get assigned a novel function (Freeling and Thomas, 2006; Freeling, 2009).

Finally, WGD leads to rapid expansion of whole gene families that can be retained to evolve and generate many genes with similar but not identical function. Thus, it is reasonable to assume that WGD particularly enables fine tuning and optimization of already existing functions.

2.5.2 State of evidence for TS-WGD causing evolutionary success and radiation

At first, we want to stress that WGDs are neither necessary for nor predictive of diversification and radiation. Although WGDs are found at the base of radiating clades such as vertebrates and flowering plants, there are also many species-rich lineages that show no signs of WGD, for instance in Coleoptera (beetles), the most diverse group of insects, consisting of over 360,000 species. Conversely lineages that are not unusually species-rich have undergone WGD, such as the Salmoniformes. Although this teleost lineage underwent an additional round of WGD, it is with 222 species comparatively species poor (Froese and Pauly, 2013). A conclusive assessment of the correlation between occurrence of WGDs and evolutionary rate or radiation requires a more complete picture on WGD across the tree of life. The

current data may very well be biased in favour of a causative role of WGD, simply due to the unequal number of species in different lineages. We expect that many more WGDs across the whole tree of life will be revealed by future genome analyses. Due to the lack of clear correlation between WGD and diversification, the sole fact that TS-WGD took place cannot be taken as evidence for it generating teleost diversity.

A way to address the question whether TS-WGD enabled teleosts to radiate is to closely look at the timing of TS-WGD and the rate of teleost diversification. A recent study estimating the timing of diversification rates in teleosts indeed revealed a prominent diversification event at the base of teleost evolution (Santini et al., 2009). This result supports a role of WGD in the diversification of teleosts. However, two additional and more recent diversification events were detected, preceding the radiation of Percomorpha and Ostariophysi, two particularly species-rich teleost clades. The delay between TS-WGD and more recent occurrence of diversification puts a causal link between TS-WGD and diversification into question. Since around 88% of species richness stems from the two more recent diversification events (Santini et al., 2009), it is suggested that it was not the primary factor generating teleost diversity. Also recent time-calibrated phylogeny of ray-finned fishes showed that the major teleost lineages originated late after TS-WGD, in the late Mesozoic and early Cenozoic (Near et al., 2012).

Interestingly, similar patterns of WGD followed by delayed radiation were found in several large groups of flowering plants that independently underwent WGD. Such lineages show greater biodiversity than lineages of flowering plants without WGD (Soltis et al., 2009). However, the major shifts in diversification did not immediately follow WGD, but took place in later emerging subclades (Smith et al., 2011). At least six species-rich families of flowering plants show a pattern of WGD followed by the emergence of both radiating and species-poor subclades (Schranz, Mohammadin, and Edger, 2012). Also the phylogenetic tree of teleosts shows such a pattern with the species-poor Elopomorpha and Osteoglossomorpha at the base and later evolving highly diverse clades such as Cyprinidae and Percomorpha. These similarities led Schranz et al. to propose that a temporal delay between WGD and radiation is a pattern generally observed and called it "time-lag model" (Schranz, Mohammadin, and Edger, 2012). Also in salmonids, a gap of 40-50 million years between WGD (which likely took place 88-103 mya) and diversification (although low in comparison to some other teleost groups) was reported (Macqueen and Johnston, 2014).

Since such patterns of delayed diversification seem to be common, they are likely not coincidental. It is possible that WGDs enable radiation long after the duplication event occurred. Indeed reciprocal gene loss and subfunction partitioning have been shown to take place long after WGD in many instances (Scannell et al., 2006; Sémon and Wolfe, 2007).

If TS-WGD did not directly drive teleost diversification, which factors did and how are they related to TS-WGD? There is not enough data available to answer these questions conclusively, but a picture is emerging. The most prominent phase marked by radiation came with the appearance of the Acanthomorpha, the most diverse group of teleosts, including the species-rich Percomorpha. The appearance of Acanthomorpha 100-150 mya (Near et al., 2012) preceded the teleost explosion, a phase when a dramatic number of new fish species emerged.

Acanthomorpha radiated in the oceans, but descended from freshwater ancestors. This transition from fresh- to saltwater is a major adaptive step due to different osmoregulatory demands of the marine environment. Once this adaptive hurdle is taken, oceans provide a rich biotope to diverge into. Therefore adaptation of Acanthomorpha to the high salinity is a likely cause of their massive oceanic radiation. Eggs principally have the same osmolarity as the maternal body fluids and are therefore hypoosmotic to sea water. If such an egg is spawned in the hyperosmotic ocean, it will suffer from osmotic water efflux. It has been shown that marine fishes increase the osmolarity of their eggs by cleaving yolk proteins, which in turn are derived from Vitellogenin (VTG). This cleavage is especially prominent in pelagic eggs, resulting in a large amount of free amino acids driving their hydration and thus making the eggs even float (Amores et al., 1998; Finn et al., 2002).

Phylogenetic analysis of the VTG family showed that VTGs are evolutionary derived from a large-lipid transfer molecule predating the origin of Bilateria (Finn and Kristoffersen, 2007). Evolution of VTG genes in vertebrates is marked by numerous duplication events (both WGD events and local duplications) as well as gene losses. Teleost VTGs can be assigned to three different types, and Acanthopterygii show a lineage specific duplication of one of the VTG genes (vtga), resulting in vtgaa and vtgab (Finn and Kristoffersen, 2007). The free amino acid pool in pelagic eggs mainly stems from VTGAA proteins (Matsubara et al., 1999), while VTGAB is essentially not degraded (LaFleur et al., 2005). vtgab thus functionally represents the ancestral state. This ancestral state of vtgab versus the derived state of vtgaa could also be confirmed by evolutionary rate analysis between the two paralogues (Finn and Kristoffersen, 2007). Finn and Kristoffersson conclude that the hydration of marine eggs and therefore the oceanic radiation of Acanthomorpha were made possible by a post-WGD event. TS-WGD only contributed indirectly by expansion of the vtg gene family that preceded the crucial duplication event in Acanthomorpha.

In salmonids, climatic changes have recently been suggested to have caused their diversification. Most salmonid lineages and species only formed in the last 10 myr, with two clades independently evolving anadromy (migratory behavior from freshwater to the oceans and back for reproduction). Macqueen and Johnston found that the shift in diversification correlates with climatic cooling, and argue that this climate change might have provided a selective advantage for anadromous behavior, since marine productivity exceeds that of freshwater in a temperate climate, providing more abundant food sources (Macqueen and Johnston, 2014). Migrating to the oceans also offered new freshwater habitats. Via estuaries, salmonids were now able to enter new river systems with new ecological demands, stimulating speciation. It has been speculated that anadromy was made possible by WGD at the base of salmonids, but clear evidence is still missing (Alexandrou et al., 2013).

In summary, there are good reasons to believe that TS-WGD has been important in generating teleost complexity. However, the time delay between TS-WGD and phases of extensive speciation suggest that TS-WGD has not been the direct factor generating teleost diversity. Ecological changes followed by adaptations also had a large impact. When environmental changes are taking place after WGD, the special modes of duplicate gene

evolution likely facilitate adaption to the new environmental conditions. Therefore, TS-WGD probably provided teleost fishes with diversification potential that can be utilized when needed, even after tens of millions of years. In other words, TS-WGD may very well set the stage for important ecological adaptations.

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2.6 Supplementary material

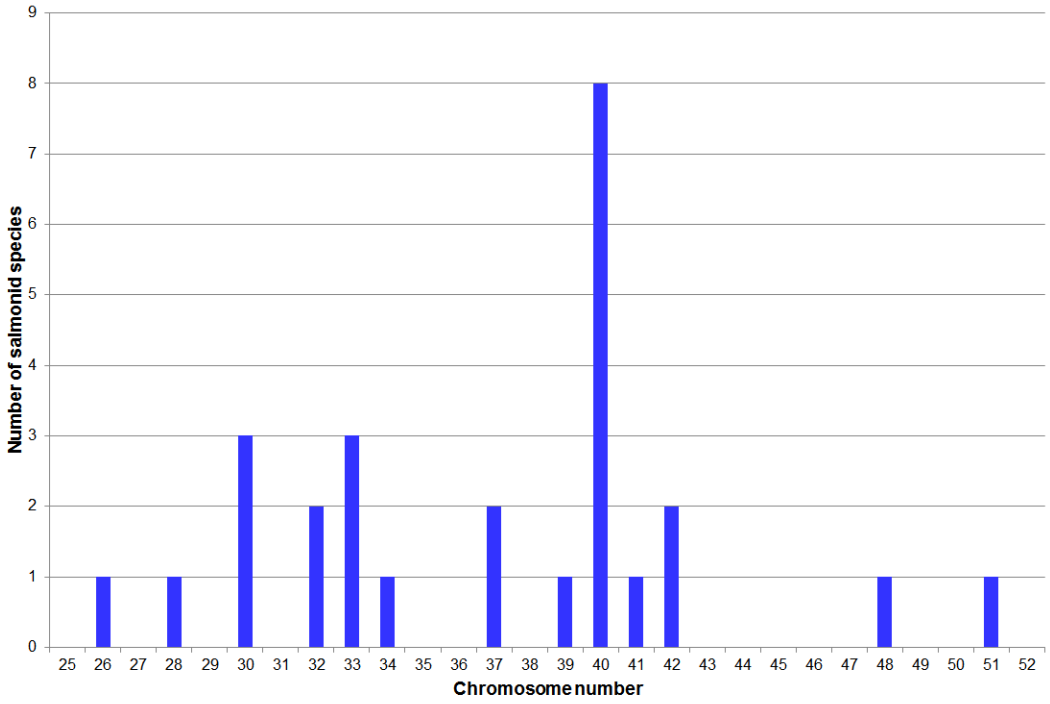

FIGURE 2.S1: Chromosome number distribution of salmonids. Haploid chromosome numbers are shown. Data is obtained from the Animal genome size database (`http://www.genomesize.com`). All 27 salmonid species' chromosome numbers available in the database were analyzed.

Chapter 3

Expression of CaBP Transcripts in Retinal Bipolar Cells of Developing and Adult Zebrafish

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3.1 Abstract

Ca^{2+}-binding proteins play important roles in neuronal function by transducing Ca^{2+} signals and thereby regulating crucial processes like synaptic signaling and neuronal development, growth and survival. The Ca^{2+}-binding protein (CaBP) subfamily is part of the vast EF-hand containing calmodulin superfamily. Eight genes encoding CaBPs have been identified in zebrafish, and many of them are expressed in specific subpopulations of retinal neurons during development. Among them, *cabp2a* and *cabp5b* have been shown to be activated in the retinal inner nuclear layer (INL). Here, we demonstrate that also their paralogues, *cabp2b* and *cabp5a*, are specifically expressed in the INL of the developing retina. By extending expression analysis of *cabp2a*, *cabp2b*, *cabp5a* and *cabp5b* to the adult retina, we reveal exclusive expression of all four genes in the INL also after retinal development is completed. Thus, our findings suggest functions of *cabp2a*, *cabp2b*, *cabp5a* and *cabp5b* in Ca^{2+} signaling in mature retinal neurons, besides a role in the developing retina.

3.2 Introduction

Ca^{2+} signalling is required for many fundamental aspects of neuronal function, such as signaling at pre- and postsynaptic sites, gene activation, growth, development and survival (Burgoyne, 2007). Ca^{2+}-binding proteins sense and transduce Ca^{2+} signals by undergoing conformational changes upon Ca^{2+} binding, and consequently regulating target proteins. The largest group of Ca^{2+}-binding proteins are structurally conserved EF-hand containing proteins belonging to the calmodulin superfamily. Within this superfamily, the vertebrate-specific CaBP subfamily comprises CaBP1, CaBP2, CaBP4 and CaBP5 in mammals (Haeseleer et al., 2000; Haynes, McCue, and Burgoyne, 2012; McCue, Haynes, and Burgoyne, 2010). In zebrafish, the CaBP subfamily is expanded to 8 members encoded by *cabp1a*, *cabp1b*, *cabp2a*, *cabp2b*, *cabp4a*, *cabp4b*, *cabp5a* and *cabp5b* (Di Donato et al., 2013), most likely due to duplicate gene retention after teleost-specific whole genome duplication (Glasauer and Neuhauss, 2014).

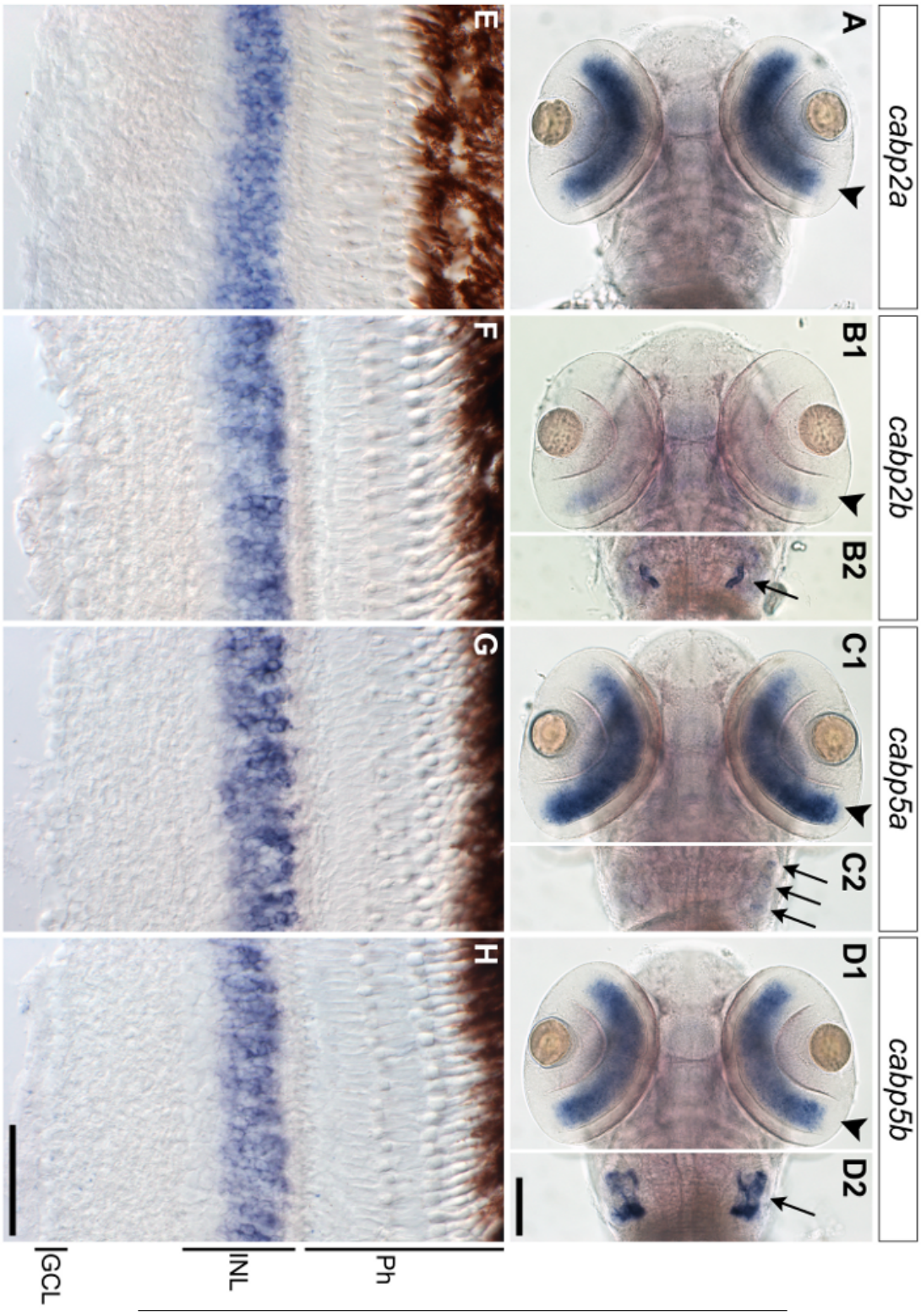
CaBPs are specifically expressed in parts of the nervous system, including the retina (Haeseleer et al., 2000; Di Donato et al., 2013; Haeseleer et al., 2004).The role of CaBP4 in retinal function is currently best understood. CaBP4, being within the retina specifically expressed in photoreceptors, has a proven role in retinal disease and photoreceptor synaptic function (Haeseleer et al., 2004; Aldahmesh et al., 2010; Khan, 2014; Littink et al., 2009; Liu et al., 2013; Maeda et al., 2005; Shaltiel et al., 2012; Zeitz et al., 2006). Functions of CaBPs expressed in second-order retinal neurons (i.e. bipolar and horizontal cells) are less well understood, although also emerging (Rieke, Lee, and Haeseleer, 2008; Sokal and Haeseleer, 2011). Bipolar cells of the mouse retina have been reported to express CaBP5 (Haeseleer et al., 2000), and *cabp2* transcripts have also been detected in this cell type (Nakajima et al., 2009). Similarly, the zebrafish orthologues *cabp2a* and *cabp5b* are expressed in retinal bipolar cells during development, while *cabp2b* expression was reported exclusively from hair cells (Di Donato et al., 2013), and *cabp5a* expression has not yet been described.

3.3 Objective

Here, we evaluate expression of *cabp5a* in the developing zebrafish retina, and extend expression analysis of *cabp2a*, *cabp2b*, *cabp5a* and *cabp5b* to the adult retina.

3.4 Results and Discussion

In order to study expression of *cabp2a*, *cabp2b*, *cabp5a* and *cabp5b* in the retina, we performed RNA *in situ* hybridization on developing whole-mount zebrafish and on adult retinal sections. In 3 day-old zebrafish larvae, all four genes studied show clear expression in the retinal inner nuclear layer (INL) (Figure 3.1: A-D). While staining for *cabp2a*, *cabp5a* and *cabp5b* results in strong labeling of the INL (Figure 3.1 A,B1,C1,D1), *cabp2b* staining is only weak in the retina (Figure 3.1: B1), also when compared to strong *cabp2b* staining observed in the inner ear (Figure 3.1: B2). Expression in the inner ear can also be detected for *cabp5a* and *cabp5b* (Figure 3.1: C2,D2). In


cabp2a
cabp2b
cabp5a
cabp5b
A
B1
B2
C1
C2
D1
D2
E
F
G
H
GCL
INL
Ph

the adult retina, all four *cabps* examined show exclusive and strong staining in the INL (Figure 3.1: G-H). Based on shape and location of labelled cell bodies, all four genes are expressed in bipolar cells (central and distal INL), and possibly also in horizontal cells (distal INL). The proximal INL, harboring amacrine and displaced ganglion cells, is devoid of staining.

Expression patterns of *cabp2a* and *cabp5b* in developing retina and ear are consistent with the findings by Di Donato et al., 2013. While expression of *cabp2b* has previously only been reported in sensory hair cells, we also detected weak expression in the retinal INL, likely reflecting low abundance of *cabp2b* transcripts in this region. Here we show for the first time expression of *cabp5a*, wich is strong in the retinal INL, and also detectable in the inner ear. Our finding that expression of *cabp2a*, *cabp2b*, *cabp5a* and *cabp5b* is maintained up to adulthood strongly suggests functions in the mature retinal INL.

So far, no reports on zebrafish CaBP function exist. Studies of CaBP5 in mammalian cell culture and mouse retina suggest an important modulatory role in retinal signaling (Rieke, Lee, and Haeseleer, 2008), possibly by regulation of voltage-gated Ca^{2+} channels (Cui et al., 2007) and a function in synaptic vesicle recruitment (Sokal and Haeseleer, 2011). Since expression of *cabp5* orthologues is conserved between mammals and zebrafish, it is well conceivable that *cabp5a* and *cabp5b* have similar tasks.

The diversity of Ca^{2+}-binding proteins expressed in the nervous system allows finely-tuned Ca^{2+} signaling through their different Ca^{2+} affinities, subcellular and cellular localizations and different binding partners, making them non-redundant regulators of neuronal signaling (Burgoyne, 2007). This and other studies (Haeseleer et al., 2000; Di Donato et al., 2013; Nakajima et al., 2009) suggest that bipolar cells exploit a repertoire of CaBPs for decoding Ca^{2+} signals, laying out the framework for further functional studies.

3.5 Conclusions

Here, we demonstrate expression of four genes encoding CaBP subfamily members in the retinal INL of zebrafish: *cabp2a*, *cabp2b*, *cabp5a* and *cabp5b*. Since all four genes are expressed in the INL of both developing and adult

FIGURE 3.1 *(preceding page)*: **Expression patterns of *cabp2a, cabp2b, cabp5a* and *cabp5b* analyzed by RNA *in situ* hybridization:** . **(A-D)** Expression in 3 day-old zebrafish larvae. **(A, B1, C1, D1)** Within the retina, all four genes are expressed in the inner nuclear layer (INL, arrowheads). Note strong labeling of the INL by *cabp2a*, *cabp5a* and *cabp5b* in contrast to comparatively weak *cabp2b* signal. **(B2, C2, D2)** In addition to the retina, *cabp2b*, *cabp5a* and *cabp5b* are also expressed in the inner ear (arrows). **(E-H)** In the adult zebrafish retina, all four genes are exclusively expressed in the INL. While central and distal regions of the INL are labelled, the proximal region remains free from staining. Scale bars correspond 100µm (D2, applies to A-D) and 50µm (E-H) (H, applies to E-H). GCL, ganglion cell layer; INL, inner nuclear layer; Ph, photoreceptor layer.

zebrafish, our findings suggest functions of *cabp2a*, *cabp2b*, *cabp5a* and *cabp5b* in Ca^{2+} signaling in mature retinal neurons, besides possible developmental roles.

3.6 Limitations

We show expression that strongly implies but does not prove function. The position of the cell bodies in the retina strongly suggests expression in bipolar and horizontal cells, but is not a formal proof.

3.7 Conjectures

The study implies a function of these CaBPs in retinal processing. The obvious next step is to genetically manipulate these genes (e.g. by CRISPR/Cas9 genome editing) and assess altered retinal function in these fish.

3.8 Funding statement

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3.9 Acknowledgements

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3.10 Ethics statement

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local authorities (Veterinäramt Zürich TV4206).

3.11 Methods

3.11.1 Fish husbandry

Zebrafish were kept at 26°C under a 14/10-hour light/dark cycle as previously described (Mullins et al., 1994). Embryos were raised at 28°C in E3 medium and staged according to development in days post fertilization (Kimmel et al., 1995). All fish used for this study were from the Tü wildtype strain.

3.11.2 Generation of DIG labeled RNA probes

Fragments of *cabp2a*, *cabp2b*, *cabp5a* and *cabp5b* coding sequence were amplified from cDNA of 5dpf zebrafish by PCR and cloned into the pCRII-TOPO vector. For primer sequences, see table 3.1. Identity of the cloned fragments was confirmed and orientation was checked by restriction digestion and sequencing. Plasmids were linearized using appropriate restriction enzymes, and *in vitro* transcription of antisense and sense RNA probes was performed using the Roche DIG-RNA Labeling Kit (Roche).

Gene	Primer name	Primer sequence
cabp2a	cabp2a_ dr_ 0030s	CAGTATGAAGAAGGGTGTCC
cabp2a	cabp2a_ dr_ 0750as	CAAAGTGCTTGTTGTCTTCC
cabp2b	cabp2b_ dr_ 0230s	CGGAAGAGATTGAAGAGCTG
cabp2b	cabp2b_ dr_ 627as	ATCCACCAATCCGTCTCC
cabp5a	cabp5a_ dr_ -0125s	ACAGACTGACGGAGGGAC
cabp5a	cabp5a_ dr_ 0560as	CTGGCTTGGCATTAGACG
cabp5b	cabp5b_ dr_ -0248s	GACACTGCTGCGTTGAGG
cabp5b	cabp5b_ dr_ 0596as	GTGACGGTTTGAATGAGTC

TABLE 3.1: Primers used for the generation of RNA probes

3.11.3 RNA *in situ* hybridization on whole mount larvae and adult retinal sections

For whole mount *in situ* hybridization, embryos were treated with 3 µM PTU (1-phenyl-2-thiourea, Sigma-Aldrich) to suppress pigmentation. 3 day-old larvae were fixed in 4% paraformaldehyde overnight at 4°C. Whole mount in situ hybridization was carried out as described (Thisse and Thisse, 2008) with modifications (Haug et al., 2013). For in situ hybridization on adult retinal sections, zebrafish were euthanized using tricaine (MS-222, Sigma-Aldrich). Eyes were excised and fixed in 4% paraformaldehyde overnight at 4°C. Tissue was washed twice in PBS for 5 minutes at room temperature, incubated in 30% sucrose overnight at 4°C, embedded in in Richard-Allan Scientific Neg-50 Frozen Section Medium (Thermo Fisher Scientific) and cryosectioned at 16 µm thickness. In situ hybridization on sections was performed as described (Haug et al., 2013). Sections were coverslipped with Kaiser's glycerol gelatin (Merck). Whole mount and section in situ hybridization experiments were carried out using antisense probes. Control experiments using sense probes resulted in no signal.

3.11.4 Image acquisition and processing

Images were acquired on a light microscope (Olympus Bx61) equipped with a Color Camera (ColorViewIII, Soft Imaging System, Olympus). Whole mount larvae were imaged in the bright field mode, and adult retinal sections in the DIC mode. Images were adjusted for brightness and contrast and figure was composed using Adobe Photoshop.

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Chapter 4

Identification of Novel Regulatory Regions Driving Expression in Bipolar Cells of the Zebrafish Retina

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4.1 Abstract

Visual processing starts at the first synapse of the retina, where each photoreceptor is generally contacted by several subtypes of bipolar cells (BPCs). BPCs serve important functions in visual processing, and different subtypes are thought to provide distinct channels for transmission of various aspects of the visual signal, such as temporal properties, contrast and chromatic composition. In the zebrafish retina, 17 morphologically distinct types of BPCs have been identified. To date, only few of them can be selectively labelled to. Transgenic techniques are powerful tools to study various properties of labelled neurons in the retina, such as connectivity, electrophysiological characteristics, response to different light stimuli, development or behavior during retinal disease. In order to establish such transgenic lines, knowledge about regulatory regions driving expression in cells of interest is of great importance. The purpose of this study was to identify novel genomic regions mediating expression in BPCs of the zebrafish retina, preferably in a subtype-specific manner. In order to do so, we cloned putative regulatory regions of genes known to be expressed in BPC of the zebrafish retina (*mglur6b*, *eaat5b*, *cabp2a*, *cabp5a*, and *cabp5b*) and tested their ability to drive expression in cabp5b in a transgenic assay. In the hope of taking advantage of the condensed nature of the *T. rubripes* genome, we also cloned and tested potential regulatory regions of orthologous fugu genes. All zebrafish genomic regions tested indeed drove expression in BPCs, while potential regulatory sequences of only one fugu candidate gene resulted in unambiguous transgene expression in the retina. Thus, we were able identify novel transgenic markers for retinal bipolar cells which have the potential to advance the understanding of bipolar cells' functions in future studies. Although different regulatory regions tested likely drive transgene expression in different subsets of BPCs, further investigation is needed to draw clear conclusions subtype-specific labelling.

4.2 Introduction

BPCs of the vertebrate retina receive direct input from photoreceptors and transmit visual information to retinal ganglion cells, which in turn project to higher-order brain regions. BPCs are the only neurons connecting light-perceiving and output cells of the retina. Additionally, they contact horizontal cells (HCs) in the outer plexiform layer (OPL) and amacrine cells (ACs) in the inner plexiform layer (IPL), making BPCs the only neurons contacting every other retinal neuron type. With this central position in the retinal connectome, BPCs serve important functions in filtering and processing of the visual signal, starting at the photoreceptor-bipolar cell synapse, the first synapse of the visual system. In the OPL, each photoreceptor is generally contacted by several types of BPCs (Li et al., 2012; Wassle et al., 2009). In mammalian retinae, slightly more than 10 BPC subtypes have been identified based on morphological criteria, taking into consideration primarily shape and position of cell bodies and axon terminals in the IPL (Wassle et al., 2009; Hartveit, 1997; Ghosh et al., 2004; Euler et al., 2014). A comparable analysis in zebrafish has revealed as many as 17 BPC subtypes (Connaughton, Graham, and Nelson, 2004). This higher number may be a consequence of the higher number of photoreceptor types found in teleost fishes compared to mammals.

The different subtypes of BPCs provide distinct channels for transmission of aspects of the visual signal, such as temporal properties, contrast and chromatic composition (Euler et al., 2014; Asari and Meister, 2012). Different functions of BPC subtypes are reflected by their morphology, connectivity and gene expression profiles.

On both the morphological and the molecular level, bipolar cells can be subdivided into ON and OFF types. OFF BPCs stratify in the distal IPL and express ionotropic AMPA/kainate type glutamate receptors on their dendrites contacting photoreceptors. As a consequence, OFF BPCs depolarize upon glutamate binding. Conversely, ON bipolar cells, stratifying in the proximal IPL, show a hyperpolarizing response to glutamate mediated by mGluR6 and its downstream effectors (Koike et al., 2010). Although the mGluR6 pathway is generally considered to mediate the full retinal ON response, studies from fish (Grant and Dowling, 1995; Grant and Dowling, 1996; Wong et al., 2004; Wong, Adolph, and Dowling, 2005; Wong, Cohen, and Dowling, 2005; Wong and Dowling, 2005; Nawy and Copenhagen, 1987), and recently also mice (Tse, Chung, and Wu, 2014), suggest an involvement of excitatory amino acid transporters (EAATs) by enabling hyperpolarization through chloride influx. One EAAT expressed on bipolar cell dendrites and possibly involved in the ON response in zebrafish is EAAT5b (Stephanie Niklaus, unpublished data).

In addition to layering of the IPL by response polarity, also temporal layering can be found. BPC terminals with fast responses stratify more centrally, and terminals with more sustained responses more peripherally in the IPL (Baden et al., 2013). The molecular basis for different kinetics of BPCs is not fully understood yet. Factors influencing response kinetics probably act in dendritic and axonal compartments of BPCs (Euler et al., 2014). Among molecules potentially tuning the temporal response profile of BPCs are members of the large EF-hand containing Ca^{2+} binding protein superfamily. Within this group, members of the CaBP subfamily have

been shown to be specifically activated in BPCs both in mammals and zebrafish (Haeseleer et al., 2000; Di Donato et al., 2013; Nakajima et al., 2009) (Glasauer and Neuhauss, submitted).

Transgenic lines provide powerful tools for studying properties of labelled retinal neurons, such as morphological and electrophysiological characteristics, Ca^{2+} dynamics, development, and their response to injury or photoreceptor degeneration. Establishment of the Tol2 transposon system in zebrafish allows highly efficient mutagenesis (Grabher and Wittbrodt, 2008; Kawakami, 2004). However, to date, only few BPC subtypes can be transgenically labelled (Schroeter, Wong, Rachel O L, and Gregg, 2006; Zhao, Ellingsen, and Fjose, 2009; Vitorino et al., 2009) (Glasauer et al., in revision).

Therefore, we attempted to identify novel regulatory regions with the ability of driving transgene expression in bipolar cells. We cloned upstream regions of genes involved in the ON response (*mglur6b* and *eaat5b*) and genes encoding selected CaBPs (*cabp2a*, *cabp5a* and *cabp5b*), and screened their ability to transgenically drive fluorescent reporter expression in the zebrafish retina. As regulatory regions can be long and interspersed with DNA sequence irrelevant to gene expression, the condensed genome of the pufferfish *Takifugu rubripes* (Aparicio et al., 2002; Brenner et al., 1993) can provide an advantage in identifying and cloning regulatory sequences. Fugu promoters have successfully been used in zebrafish transgenesis (Zou et al., 2006; Udvadia, 2008; Kato et al., 2015; Woolfe et al., 2005). Therefore, we cloned potential regulatory regions not only from zebrafish, but also from fugu.

We tested a total of 12 different putative regulatory regions from 4 zebrafish and 3 fugu genes. All zebrafish genomic regions tested indeed drove expression in BPCs, while among fugu putative regulatory sequences, only *cabp5a* upstream sequences unambiguously drove transgene expression in BPCs. In summary, we successfully identified novel transgenic markers for retinal bipolar cells with the potential to advance understanding of bipolar cells and their subtypes in future studies.

4.3 Methods

4.3.1 Fish maintenance and breeding

Zebrafish were kept at 26°C as previously described (Mullins et al., 1994). Embryos were raised at 28°C in E3 medium and staged according to development in days post fertilization (dpf) (Kimmel et al., 1995) All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local authorities (Veterinäramt Zürich TV4206).

4.3.2 Generation of Tol2 transgenesis constructs

Potential regulatory regions of genes expressed in bipolar cells were PCR amplified using a proof-reading Polymerase (Phusion High Fidelity DNA Polymerase, New England Biolabs). The following potential regulatory regions were successfully amplified from zebrafish gDNA: zebrafish *eaat5b* 3.4 kb (primers Dr_ eaat5b_ -3381_ fw_ attB4 and Dr_ eaat5b_ -0019_ fw_

Species	Potential regulatory region of	Primer name	Primer sequence (without attB sites)
zebrafish	*eaat5b*	Dr_ eaat5b_ -6560_ fw_ attB4	GGTATTTGGAGTGGTACAGC
		Dr_ eaat5b_ -3381_ fw_ attB4	TCAAGCCACTATCAAACTTGG
		Dr_ eaat5b_ -0019_ fw_ attB1	AAACACCAGCCACACTAGC
		Dr_ eaat5b_ -6689_ fw	CACAGGCATTTGTAGCTTCG
		Dr_ eaat5b_ 0063_ rev	CAAAACCGAGAGGATCAGC
	cabp2a	Dr_ cabp2a_ -2650_ fw_ attB4	GATTGCTACTACCACATTATCTCC
		Dr_ cabp2a_ -0003_ rev_ attB1	TGCCTCTTTTAGATGGTTTCG
		Dr_ cabp2a_ 1785_ rev_ attB1	CATTAGGTACCAGAGAAGTTCC
	cabp5b	Dr_ cabp5b_ -4843_ fw_ attB4	GATTACTGGGCTACTCTTATGC
		Dr_ cabp5b_ 0029_ rev_ attB1	TCTTTTGAGTTTCTTCTGAGCC
fugu	*mglur6b*	Tr_ mglur6b_ -1911_ fw_ attB4	GAGGATGGCTTAGAGAAATCTG
		Tr_ mglur6b_ -0001_ rev_ attB1	GGTGATGACCAGCACAGG
		Tr_ mglur6b_ 2628_ rev_ attB1	GTTCAGTGATGGCAAAATAGG
	cabp5a	Tr_ cabp5a_ -3350_ fw_ attB4	TAGGGCTGCAGCATATGTG
		Tr_ cabp5a_ -2397_ fw_ attB4	GCAAATACAGACAGCTTTGC
		Tr_ cabp5a_ 0019_ rev_ attB1	CAGAGTTTGATGTGAAATGTAACG
	cabp5b	Tr_ cabp5b_ -0644_ fw_ attB4	AGATGAGGGTTCTAATACTACGG
		Tr_ cabp5b_ -0010_ rev_ attB1	CTCAGTCAAGAGCTTGG
		Tr_ cabp5b_ 0602_ rev_ attB1	ACAAAAGCTTCACGCAGC

TABLE 4.1: **Primers used for amplifying potential regulatory regions**. Numbers in primer names indicate nucleotide positions relative to translational start sites. Forward primers used in BP reactions carry attB4 site (GGG GACAACTTTGTATAGAAAAGTTG) and reverse primers attB1 site extensions (GGGGACTGCTTTTTTGTACAAAC TTG) at their 5′ ends.

attB1), zebrafish *cabp2a* 2.7 kb (primers Dr_ cabp2a_ -2650_ fw_ attB4 and Dr_ cabp2a_ 1785_ rev_ attB1) and 4.4 kb (primers Dr_ cabp2a_ -2650_ fw_ attB4 and Dr_ cabp2a_ 1785_ rev_ attB1), and zebrafish *cabp5b* 4.9 kb (primers Dr_ cabp5b_ -4843_ fw_ attB4 and Dr_ cabp5b_ 0029_ rev_ attB1). Zebrafish *eaat5b* 6.5 kb was amplified from zebrafish gDNA by nested pcr with primers Dr_ eaat5b_ -6689_ fw and Dr_ eaat5b_ 0063_ rev in the first and primers Dr_ eaat5b_ -6560_ fw_ attB4 and Dr_ eaat5b_ -0019_ fw_ attB1 in the second round of PCR. Fugu *mglur6b* 1.9 kb (primers Tr_ mglur6b_ -1911_ fw_ attB4 and Tr_ mglur6b_ -0001_ rev_ attB1) and 4.5 kb (primers Tr_ mglur6b_ -1911_ fw_ attB4 and Tr_ mglur6b_ 2628_ rev_ attB1) were amplified from *Takifugu rubripes* BAC clone 228 F19 (Source BioScience). Fugu *cabp5a* 2.4 kb (primers Tr_ cabp5a_ -2397_ fw_ attB4 and Tr_ cabp5a_ 0019_ rev_ attB1) and 3.4 kb (primers Tr_ cabp5a_ -3350_ fw_ attB4 and Tr_ cabp5a_ 0019_ rev_ attB1), fugu *cabp5b* 0.6 kb (primers Tr_ cabp5b_ -0644_ fw_ attB4 and Tr_ cabp5b_ -0010_ rev_ attB1) and 1.2 kb (primers Tr_ cabp5b_ -0644_ fw_ attB4 and Tr_ cabp5b_ 0602_ rev_ _ attB1) were amplified from fugu gDNA. Attempts to amplify fugu *eaat5* and zebrafish *cabp5a* regulatory regions resulted in no or only small amounts of PCR product that could not be successfully cloned. Recombination reactions were performed according to the Gateway™ Three-Fragment Vector Construction Kit (Invitrogen, Life Technologies). Briefly, a BP recombination reaction was

performed to integrate the amplified fragment into pDONRP4-P1R (Invitrogen), resulting in a 5' entry clone suitable for Multisite Gateway cloning. Restriction and partial sequencing confirmed identitiy and correct insertion of upstream elements. To create Tol2 expression constructs , we performed a multisite LR reactions of our newly created 5' entry clones or a 5' entry clone with 4.8 kb upstream sequence of zebrafish *mglur6b* (Glasauer et al., in revision), together with pME-EGFPCAAX (middle entry clone), p3E-polyA (3' entry clone) and pDestTol2CG2 (destination vector; all vectors from Tol2 kit, (Kwan et al., 2007). Again, correct assembly of the resulting vectors was checked and confirmed by restriction and partial sequencing.

4.3.3 Transposase mRNA synthesis

pCS2FA-transposase plasmid (Kwan et al., 2007) was linearized with NotI. Capped RNA was transcribed using mMessage mMachine SP6 kit (Ambion), phenol:chloroform extracted and precipitated using isopropanol according to the manufacturer's instructions.

4.3.4 Microinjections and screening of injected fish

11 to 44 pg plasmid and 12 to 25 pg transposase mRNA were co-injected into one-cell stage zebrafish embryos. After gastrulation but before 1dpf, embryos were treated with 3 µM PTU (1-phenyl-2-thiourea, Sigma-Aldrich) to suppress pigmentation. Embryos and larvae were viewed under a fluorescent Stereomicroscope (Olympus MVX10) equipped with a color camera (ColorViewIII, Soft Imaging System, Olympus). 3 dpf and older larvae were anesthetized with Tricaine (MS-222, Sigma-Aldrich). In a typical experiment, EGFP expression in the heart (transgenesis marker) could be seen in 75-100% of the injected embryos from around 1 dpf onwards. Larvae were scored for transgene expression in the eyes at 5 dpf. For acquiring live images, anesthesized larvae were mounted in 1% low melting temperature agarose (Nu Sieve GTG agarose, Lonza) in E3 medium and covered with E3 medium containing Tricaine.

4.3.5 Immunohistochemistry

5 dpf larvae showing expression of the cmlc2:EGFP transgenesis marker were fixed overnight in 4% paraformaldehyde at 4°C. Section immunohistochemistry was carried out as described previously (Rinner et al., 2005), except that tissue was embedded in Richard-Allan Scientific Neg-50 Frozen Section Medium (Thermo Fisher Scientific) and cryosectioned at 16 µm thickness. Primary antibodies were chicken anti-GFP (A10262, Molecular Probes, Life Technologies; 1:500) and mouse anti-PKCalpha MC5 (NB200-586: Novus Biologicals; 1:500). Goat anti-chicken and anti-mouse secondary antibodies conjugated to Alexa 488 or 568 fluorophores (Molecular Probes, Life Technologies), were used at dilutions 1:500 - 1:1000. BODIPY TR Methyl Ester (Molecular Probes, Life Technologies; 1:300) was applied for 20 min after secondary antibodies, and sections were coverslipped with Mowiol (Polysciences) mounting medium containing DABCO (Sigma-Aldrich). Images were acquired on a Leica HCS LSI confocal microscope equipped with a 40x oil objective (Leica Microsystems). Images were adjusted for brightness and contrast using ImageJ and figures were composed with Adobe Photoshop.

4.4 Results

4.4.1 Identification of potential regulatory regions

mglur6b, *eaat5b*, *cabp2a*, *cabp5a* and *cabp5b* have been previously shown to be expressed in bipolar cells of the zebrafish retina during both larval stages and adulthood (Huang et al., 2012; Di Donato et al., 2013) (Glasauer and Neuhauss, in revision, Stephanie Niklaus, unpublished data), making their regulatory regions good candidates to transgenically label and study BPC populations throughout life. In order to identify orthologs of these zebrafish candidate genes in *T. rubripes*, we performed Blast searches on the fugu genome assembly (FUGU 4.0, Ensembl Genome Browser) and constructed maximum likelihood phylogenetic trees (Phylogeny.fr, RRID:SCR_010266, not shown) to unambiguously identify the closest homologs to our zebrafish candidate genes. Our analysis revealed clear fugu orthologs of all zebrafish candidate genes except *eaat5b*. Lack of *eaat5b* in fugu has been noted previously (Gesemann et al., 2010).

For choosing potential regulatory regions of zebrafish and fugu candidate genes, we took into consideration the following factors: Proximity to the translational or, if annotated, transcriptional start site, conservation to other fish species, and repetitiveness. Conservation of non-coding sequence to other fish species can indicate functionality, whereas repetitive sequence often reflects non-functionality, at least in terms of regulating gene expression. Information on both conservation and repetitiveness were retrieved from the UCSC genome browser, and were taken into consideration for setting 5′ and 3′ ends of potential regulatory sequences. Fugu genomic regions around translational start sites of our candidate genes were generally much poorer in repetitive sequence and showed more conservation to other fish species than homologous regions of the zebrafish genome, as exemplified by fugu and zebrafish mglur6b genes (Figure 4.1).

4.4.2 Zebrafish regulatory regions efficiently drive transgene expression in BPCs

We successfully amplified and cloned 12 potential regulatory regions from 4 zebrafish and 3 fugu genes (see Methods, page 58), generating Tol2 trangenesis vectors with membrane-tagged EGFP (EGFPCAAX) as reporter gene (Figure 4.1B). EGFPCAAX was chosen because of its ability to label very thin neurite extensions, a property potentially useful for future studies of detailed cell morphology and connectivity.

In order to test the ability of potential regulatory sequences to drive transgene expression in bipolar cells of the retina, Tol2 constructs were microinjected into one-cell stage zebrafish embryos, yielding zebrafish with mosaic transgene expression. Injection of all 6 constructs harboring potential regulatory regions from the zebrafish genome resulted in fluorescent reporter expression in the eyes by 5 dpf (Table 4.2) detectable by live imaging under a fluorescent stereomicroscope. The 4.8 kb upstream region of *mglur6b* drove clearly visible reporter expression in 20% of fish expressing the transgenesis marker cmlc2:EGFP, whereas regulatory regions of *eaat5b*, *cabp2a* and *cabp5b* consistently led to transgenic eye expression in more than 50% of transgenic zebrafish. One construct driving particularly clear and

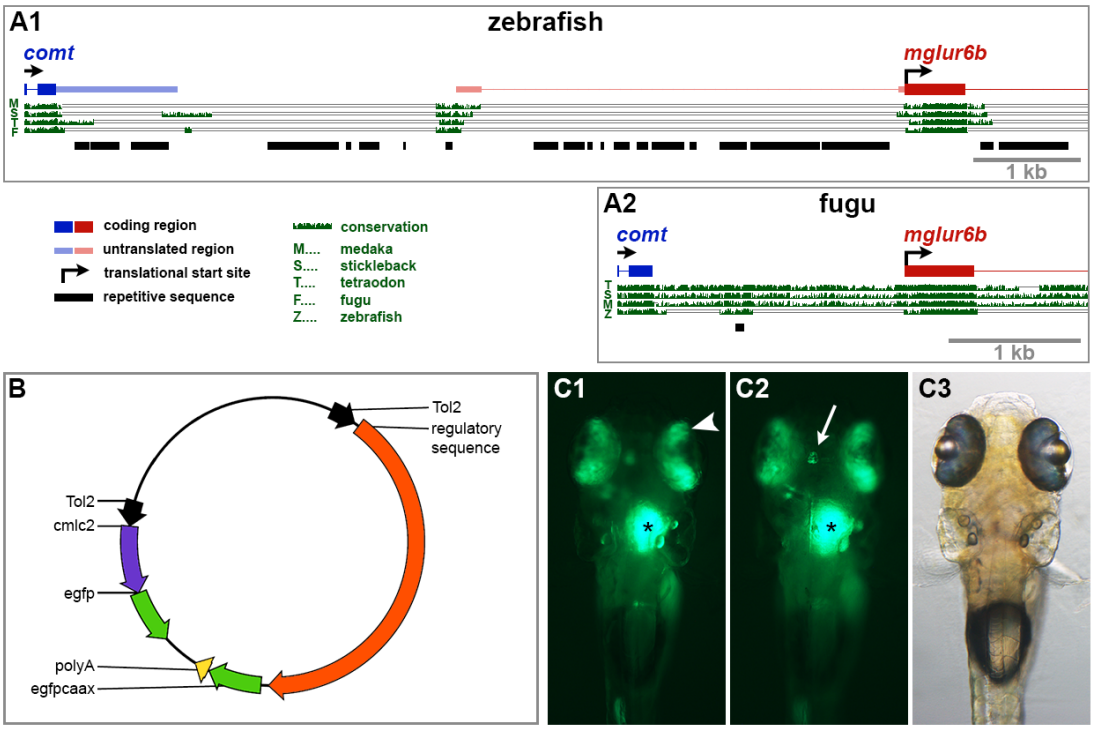


FIGURE 4.1: **(A) Comparison of zebrafish and fugu genomic regions around mglur6b translational start sites**. In comparison to zebrafish **(A1)**, the fugu locus **(A2)** exhibits shorter distance between the neighboring genes *mglur6b* and *comt*, fewer repetitive and more conserved sequence. Images show modified snapshots from the UCSC genome browser (genome.ucsc.edu). "Repetitive sequence" corresponds to repeat masker track, and "conservation" to Multiz Alignment and Conservation tracks of zebrafish (Zv9/danRer7 assembly) and fugu (FUGU5/fr3) genome assemblies. **(B)** Generalized vector map of Tol2 transgenesis constructs generated. **(C)** Example of a 6 days post fertilization (dpf) zebrafish injected with Dr_eaat5b(6.5kb):EGFPCAAX construct. Live imaging under a fluorescence stereomicroscope reveals strong fluorescence in the retina (arrowhead). Also note the signal in the epiphysis (arrow) and expression of transgenesis marker cmlc2:EGFP (asterisks).

strong reporter expression was the Dr_ eaat5b(6.5kb):EGFPCAAX plasmid (Figure 4.1C).

In order to identify which cell types of the retina express the transgenes, we performed immunohistochemistry on 5 dpf larval sections. Injection of all zebrafish constructs resulted in fluorescence of cells with typical bipolar cell morphology and position within the INL (Figure 4.2 A-F). Injection of constructs containing zebrafish *eaat5b* upstream regions resulted in exclusive staining of cell bodies in the INL (Figure 4.2 B,C), while injection of the other zebrafish constructs additionally led to weak (*cabp2a* regulatory regions, Figure 4.2 D,E) or strong (*mglur6b* and *cabp5b* regulatory regions Figure 4.2 A,F) signal in the ganglion cell layer (GCL).

4.4.3 Fugu putative regulatory regions drive weak or sparse expression in the zebrafish retina

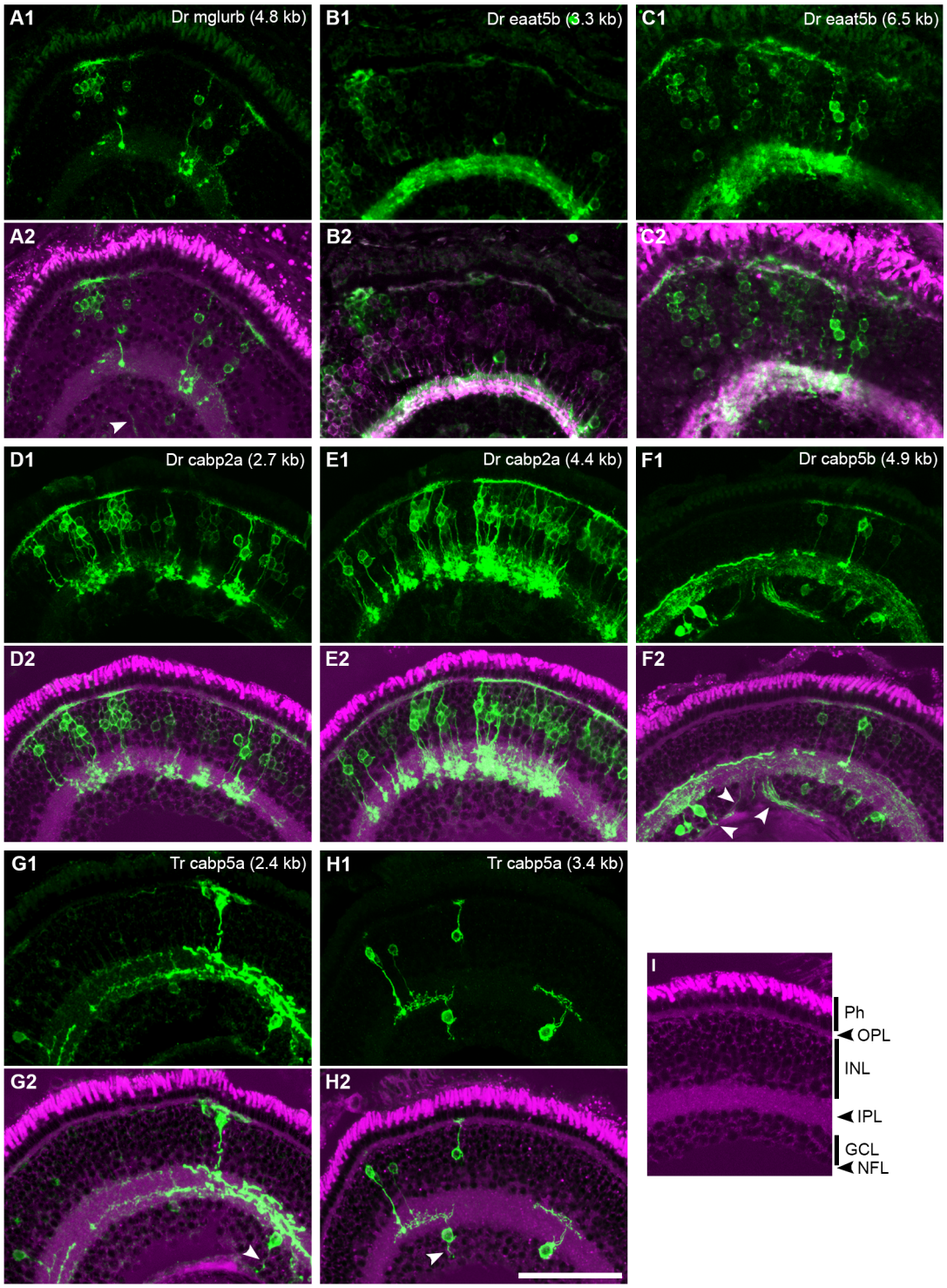
No unambiguos fluorescence in the eyes could be observed by live imaging upon injection of any of the fugu constructs, despite clear expression of the transgenesis marker (Table 4.2). In order to detect the potential presence of weak transgene expression in a more sensitive way, we also performed immunohistochemistry on zebrafish larvae injected with fugu constructs.

Larvae injected with fugu *mglur6b* and *cabp5b* constructs showed very weak signal resembling cell bodies in the INL or bipolar cell axon terminals in the IPL (not shown), likely reflecting very low expression of these transgenes in BPCs. In constrast, immunohistochemistry on fugu *cabp5a* construct injected fish revealed surprisingly clear staining of few bipolar and ganglion cells (Figure 4.2 G,H). This finding indicates that we failed to detect transgene expression by live imaging for fugu *cabp5a* constructs not due to weak, but rather sparse transgene expression possibly restricted to only few bipolar and ganglion cell subtypes.

4.5 Discussion

In this study, we attempted to identify genomic regions driving expression specifically in BPCs of the retina, preferably in a subtype-specific manner. The knowledge of such regulatory regions will be useful to establish transgenic zebrafish lines advancing the understanding of specific functions of BPCs and BPC subtypes. To achieve this, we selected candidate genes with known expression in zebrafish BPCs, cloned their potential regulatory regions and tested their ability to drive transgene expression in BPCs. In the hope of taking advantage of the condensed nature of the *T. rubripes* genome, we also cloned and tested potential regulatory regions of orthologous fugu genes.

In summary, all zebrafish genomic regions tested indeed drove expression in bipolar cells, while potential regulatory sequences of only one fugu candidate gene resulted in unambiguous transgene expression in the retina. Regulatory regions of two zebrafish genes drove transgene expression exclusively or predominantly in retinal bipolar cells (zebrafish *eaat5b* and *cabp2a* regulatory regions), whereas regulatory regions of three more genes (zebrafish *mglur6b*, zebrafish *cabp5b* and fugu *cabp5a*) resulted in clear expression in both BPCs and cells of the GCL. In conclusion, we were successful


A1
Dr mglurb (4.8 kb)
B1
Dr eaat5b (3.3 kb)
C1
Dr eaat5b (6.5 kb)
A2
B2
C2
D1
Dr cabp2a (2.7 kb)
E1
Dr cabp2a (4.4 kb)
F1
Dr cabp5b (4.9 kb)
D2
E2
F2
G1
Tr cabp5a (2.4 kb)
H1
Tr cabp5a (3.4 kb)
G2
H2
I
Ph
OPL
INL
IPL
GCL
NFL

in finding regulatory regions that can serve as tools for labeling BPCs in transgenic lines.

4.5.1 Zebrafish regulatory elements largely recapitulate endogenous expression of candidate genes

Injection of any of the constructs harboring zebrafish potential regulatory regions resulted in transgene expression in bipolar cells, demonstrating that cis-regulatory elements essential for expression in BPCs are contained in all our cloned putative regulatory regions, which were between 2.7 kb and 6.5 kb long.

The zebrafish *eaat5b* transgenic constructs drove transgene expression only in the INL of injected larvae, matching endogenous *eaat5b* expression (Stephanie Niklaus, unpublished data). Upon injection of constructs containing zebrafish *mglur6b* and *cabp5b* regulatiory regions, we found clear additional transgene expression in the GCL. Thus, also expression driven by the *mglur6b* construct recapitulates endogenous gene expression (Huang et al., 2012) (see also Glasauer et al., in revision). In contrast, as *cabp5b* mRNA was not reported from the GCL (Di Donato et al., 2013) (Glasauer and Neuhauss, submitted) the *cabp5b* regulatory sequence cloned appears to drive ectopic transgene expression in ganglion cells. It seems likely that the cloned 4.9 kb fragment of zebrafish *cabp5b* regulatory sequence lacks elements relevant for INL specific expression, such as repressors inhibiting *cabp5b* activation in the GCL. Similarly, also injection of our zebrafish cabp2a constructs resulted in signal within the GCL, although rather weak. It is therefore not clear whether these constructs drive broader than endogenous mRNA expression, or whether low abundance of *cabp2a* transcripts was not detected in RNA expression studies.

Whenever we cloned two genomic regions of different lengths for a candidate gene, EGFP expression patterns were essentially the same for

FIGURE 4.2 *(preceding page)*: **Analysis of cell types labelled after injection of transgenic constructs. (A-F)** All transgenic constructs harboring zebrafish regulatory regions drive EGFPCAAX expression in retinal bipolar cells (BPCs) of the inner nuclear layer (INL). The *mglur6b* and *cabp5b* constructs **(A, F)** additionally drive clear fluorescence in cells of the ganglion cell layer (GCL) and processes extending to the nerve fiber layer (white arrowheads), demonstrating expression in retinal ganglion cells (GCs). Weak signal in the GCL can also be observed after injecting *cabp2a* constructs **(D,E)**. **(G,H)** Injection of fugu *cabp5a* constructs results in labelling of BPCs, GCs and possibly also amacrine cells. **(I)** Visualization of retinal layers by BODIPY staining for better orientation. All images show 5dpf larval sections subjected to immunohistochemistry. Green, EGFPCAAX; magenta, BODIPY **(A, C-I)** or PKCα **(B)**. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NFL, nerve fiber layer; OPL, outer plexiform layer; Ph, photoreceptor layer. Scale bar corresponds to 50 µm.

species	Regulatory region	Potential regulatory region of	length	Eye expression visible in live imaging (5dpf)	Eye expression determined by IHC (5dpf)	
					INL	GCL
zebrafish	Dr_ mglur6b(4.8kb):EGFPCAAX	*mglur6b*	4.8 kb	20% (45/225)*	+	+
	Dr_ eaat5b(3.3kb):EGFPCAAX	*eaat5b*	3.4 kb	73% (168/229)	+	-
	Dr_ eaat5b(6.5kb):EGFPCAAX		6.5 kb	56% (181/322)	+	-
	Dr_ cabp2a(2.7kb):EGFPCAAX	*cabp2a*	2.7 kb	93% (13/14)	+	(+)
	Dr_ cabp2a(4.4kb):EGFPCAAX		4.4 kb	63% (53/84)	+	(+)
	Dr_ cabp5b(4.9kb):EGFPCAAX	*cabp5b*	4.9 kb	55% (35/64)	+	+
fugu	Tr_ mglur6b(1.9kb):EGFPCAAX	*mglur6b*	1.9 kb	0% (0/27)	(+)	-
	Tr_ mglu6b(4.5kb):EGFPCAAX		4.5 kb	0% (0/40)	(+)	-
	Tr_ cabp5a(2.4kb):EGFPCAAX	*cabp5a*	2.4 kb	0% (0/22)	+	+
	Tr_ cabp5a(3.4kb):EGFPCAAX		3.4 kb	0% (0/11)	+	+
	Tr_ cabp5b(0.6kb):EGFPCAAX	*cabp5b*	0.6 kb	0% (0/32)	(+)	-
	Tr_ cabp5b(1.2kb):EGFPCAAX		1.2 kb	0% (0/50)	(+)	-

TABLE 4.2: **Summary of transgenic constructs tested.** Percentages represent numbers of fish with visible EGFP or EGFPCAAX expression in their eyes divided by number of fish showing expression of the cmlc2:EGFP transgenesis marker. GCL, ganglion cell layer; IHC, immunohistochemistry; INL, inner nuclear layer. *Data from Wäger, 2012.

both constructs, indicating that no regulatory elements conveying specificity to retinal layers were contained in parts exclusively contained in the longer genomic fragments. However, made the interesting observation that larvae injected with the Dr_ eaat5b(6.5kb):EGFPCAAX construct generally exhibited stronger transgene expression than larvae injected with the Dr_ eaat5b(3.3kb):EGFPCAAX construct. Therefore, the region exclusively included in the 6.5 kb fragment might contain enhancer elements increasing *eaat5b* expression.

4.5.2 Lower success rate of BPC labelling by fugu putative regulatory sequences

Generally, we found genomic regions around translational start sites of our fugu candidate genes to contain less repetitive and more conserved sequence than their zebrafish counterparts. This observation is consistent with the condensed nature of the pufferfish genome (Aparicio et al., 2002; Brenner et al., 1993). However, higher degree of conservation to other fish species (medaka, tetraodon and stickleback) is probably partly due to the fact that these species are more closely related to fugu than to zebrafish.

Although all fugu constructs tested were principally able to activate transgene expression in BPCs, injection of plasmids harboring *mglur6b* and *cabp5b* potential regulatory elements only resulted in weak signal. Only plasmids containing fugu *cabp5a* upstream regions were clearly able to drive expression in BPCs and also in retinal ganglion cells. Interestingly, expression appeared sparse but strong; making the fugu *cabp5a* constructs good candidates to label only few BPC subtypes.

Possible explanations for only detecting weak transgene expression upon injection of fugu *mglur6b* and *cabp5b* constructs are multiple. They include incomplete cloning of critical enhancer elements, non-conserved expression patterns between orthologous genes, and divergent evolution of transcriptional regulation. Divergence in transcriptional regulation can occur without divergence in expression patterns (reviewed in Payne and Wagner, 2015; Weirauch and Hughes, 2010) and may have resulted in altered affinities of transcription factors controlling *mglur6b* and *cabp5b* expression between fugu and zebrafish. Furthermore, even different transcription factors may regulate expression of orthologous fugu and zebrafish genes, since such divergence has been observed within several phyla (Schmidt et al., 2010; Moses et al., 2006; Borneman et al., 2007).

4.5.3 BPC subtype specific labelling remains speculative

Although we could show that regulatory regions of 5 genes tested can drive expression in zebrafish BPCs, it is not clear yet whether they do so in a subtype-specific manner. From our immunohistochemistry experiment, it seems that regulatory elements of zebrafish *mglur6b*, *eaat5b* and *cabp2a* drive rather broad expression when compared to rather sparse labeling of the INL found after injection of zebrafish *cabp5b* and fugu *cabp5a* constructs. We also noted that many of the BPCs labelled after injection of zebrafish *cabp2a* constructs have big axon terminals in the proximal IPL (Figure 4.2 D,E), while the BPCs labelled after injection of zebrafish *cabp5b* and fugu *cabp5a* constructs rather seem to stratify in the central and distal IPL (Figure 4.2 F-H).

However, low sample size in this experiment (n $\approx$ 3-10) and mosaicism of the injected larvae make statements about the number of labelled subtypes difficult. Only stable transgenic lines will allow to draw clear conclusions about the identity and number of labelled BPC subtypes.

4.5.4 Conclusions

In summary, we tested regulatory regions of 4 zebrafish and 3 fugu genes for their ability to drive transgene expression in BPCs of the zebrafish retina. We found regulatory regions of all 4 zebrafish genes, but of only one fugu gene to drive clear expression in bipolar cells. Therefore, although cloning of fugu regulatory regions is less tedious due to the compact nature of the fugu genome, the lower success rate for putative regulatory regions abrogated this advantage in our study. Nonetheless, we were able identify novel transgenic markers for retinal bipolar cells which have the potential to advance the understanding of bipolar cell subtypes and their functions in future studies.

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Chapter 5

Mglur6b:EGFP Transgenic Zebrafish Suggest Novel Functions of Metabotropic Glutamate Signaling in Retina and Other Brain Regions

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Personal contribution: Study concept and design, performing all experiments except establishing the line up to F1 generation, data acquisition and analysis, preparation of all figures, writing and revision of the manuscript

5.1 Abstract

Metabotropic glutamate receptors (mGluRs) are mainly known for regulating excitability of neurons. However, mGluR6 at the photoreceptor-ON bipolar cell synapse mediates sign inversion through glutamatergic inhibition. Although this is currently the only confirmed function of mGluR6, other functions have been suggested. Here, we present *Tg(mglur6b:EGFP)zh1*, a new transgenic zebrafish line recapitulating endogenous expression of one of the two *mglur6* paralogues in zebrafish. Investigating transgene as well as endogenous *mglur6b* expression within the zebrafish retina indicates that EGFP and *mglur6b* mRNA are not only expressed in bipolar cells, but also in a subset of ganglion and amacrine cells. The amacrine cells labeled in *Tg(mglur6b:EGFP)zh1* constitute a novel cholinergic, non-GABAergic, non-starburst amacrine cell type described for the first time in teleost fishes. Apart from the retina, we find transgene expression in subsets of periventricular neurons of the hypothalamus, Purkinje cells of the cerebellum, various cell types of the optic tectum and mitral/ruffed cells of the olfactory bulb. These findings suggest novel functions of *mGluR6* besides sign inversion at ON bipolar cell dendrites opening up the possibility that inhibitory glutamatergic signaling may be more prevalent than currently thought.

5.2 Introduction

Glutamate is the major excitatory neurotransmitter in the central nervous system, including the retina. Both ionotropic and metabotropic receptors exert wide-spread functions of glutamate in neuronal signaling. Ionotropic glutamate receptors (iGluRs) are nonselective cation channels that directly generate an excitatory signal upon glutamate binding. In contrast, Metabotropic glutamate receptors (mGluRs) modulate ion channels indirectly through several intracellular signaling cascades. mGluRs can be divided into three types based on pharmacology, signaling pathways activated and similarity in amino acid sequence (Conn and Pin, 1997). Type I mGluRs are generally postsynaptic and stimulate phospholipase C through Gq/11 protein signaling. Conversely, type II and type III mGluRs predominantly lie presynaptically where they activate Gi/o proteins, thereby inhibiting cAMP production and consequently reducing protein kinase A activity.

In addition, type III mGluRs are known as auto- and heteroreceptors regulating neurotransmitter release, a function that mostly relies on cAMP independent regulation of ion channels. In particular, type III mGluRs inhibit Ca^{2+} channels and activate K^{+} channels (Mercier and Lodge, 2014). Moreover, they have been proposed to regulate neurotransmitter release by directly acting on the exocytotic machinery (Anwyl, 1999). There are four members of type III mGluRs in mammals: mGluR4, mGluR6, mGluR7 and mGluR8. Among them, mGluR6 stands out due to its atypical postsynaptic localization. In the mammalian retina, it mGluR6 was reported to be exclusively localized to dendritic tips of ON bipolar cells (Nomura et al., 1994). ON bipolar cells, in contrast to sign-preserving OFF bipolar cells, reverse the sign of the photoreceptor signal due to mGluR6 signaling: In darkness, photoreceptors are depolarized and continuously release glutamate, which is sensed by mGluR6 at the postsynapse. Upon glutamate binding, mGluR6 interacts with the effector G protein Goα (Nawy, 1999; Dhingra et al., 2000), which mediates closure of the constitutively active cation channel TRPM1 (Koike et al., 2010; Morgans et al., 2009), leading to hyperpolarization of the ON bipolar cell. Because of its crucial role in retinal signaling, mutations in human *MGLUR6* cause congenital stationary night blindness, accompanied by typical loss of the ON response in the electroretinogram (Dryja et al., 2005). Consistently, absence of mGluR6 results in impaired ON signaling in animal models (Huang et al., 2012; Masu et al., 1995; Zeitz et al., 2005). Besides mGluR6 playing a direct role in synaptic transmission, it is also necessary for postsynaptic localization of proteins involved in the ON signaling cascade (Cao et al., 2009; Morgans et al., 2007) and the $Ca2^{2+}$ channel subunit Cacna1s (Tummala et al., 2014).

While the role of mGluR6 in ON bipolar cells has been well described, reports suggesting additional functions of mGluR6 are scarce. In adult rodents, *mglur6* transcripts were never found in healthy retinal ganglion cells, but expression was activated in juvenile ganglion cells and adult ganglion cells after optic nerve injury in rats (Tehrani, Wheeler-Schilling, and Guenther, 2000). In transgenic mice that express enhanced green fluorescent protein (egpf) under the *mglur6* promoter (Dhingra et al., 2008), EGFP was detected in amacrine cells and retinal ganglion cells during early embryonic development (Morgan et al., 2006) as well as in certain brain areas, such as accessory olfactory bulb. Human retinal ganglion cells also express

MGLUR6, (Klooster et al., 2011) and *mglur6* transcripts were even identified outside the mouse nervous system (Vardi et al., 2011).

Work in zebrafish has clearly shown that the two *mglur6* paralogs present in the zebrafish genome, *mglur6a* and *mglur6b*, are not only expressed in bipolar cells, but also in the proximal inner nuclear layer, retinal ganglion cells and various additional brain regions (Haug et al., 2013; Huang et al., 2012).

Here, we present a new transgenic zebrafish line, in which an *mglur6b* upstream regulatory region drives *EGFP* expression. Using this line, we could identify a novel cholinergic amacrine cell type in the retina and demonstrate a potential role of mGluR6 signaling in neurons within a number of brain regions, including projection neurons of the olfactory bulb.

5.3 Materials and Methods

5.3.1 Fish maintenance and breeding

Zebrafish were kept at 26°C under a 14/10-hour light/dark cycle as previously described (Mullins et al., 1994). Embryos were raised at 28°C in E3 medium and staged according to development in days post fertilization (dpf) (Kimmel et al., 1995). All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local authorities (Veterinäramt Zürich TV4206).

5.3.2 Creation of *Tg(mglur6b:EGFP)* construct

A 4.8 kb fragment upstream of *mglur6b*, ending at position -16 relative to the *mglur6b* translational start site, was PCR amplified from BAC clone DKEY-13A21 (Source BioScience) using a proof-reading Polymerase (Phusion High-Fidelity DNA Polymerase, FINNZYMES). PCR primers with attB4 (forward primer) and attB1 (reverse primer) sites added to 5′ ends were used to allow Gateway cloning of the amplified fragment (forward primer: GGGGACAACTTTGTATAGAAAAGTTGACAGGCTACGGATATTTCA GTTC; reverse primer: GGGGACAAGTTTGTACAAAAAAGCAGGCTA GCGGCTCGTTGTTCTTCTAC).

Recombination reactions were performed according to the Gateway™ Three-Fragment Vector Construction Kit (Invitrogen, Life Technologies). Briefly, a BP recombination reaction was performed to integrate the amplified fragment into pDONRP4-P1R (Invitrogen), resulting in a 5′ entry clone suitable for Multisite Gateway cloning. Restriction and partial sequencing confirmed identity and correct insertion of the *mglur6b* upstream element. To create the *Tg(mglur6b:EGFP*) expression construct (Figure 5.1A), we performed a multisite LR reaction of our 5′ entry clone, pME-EGFP (middle entry clone), p3E-polyA (3′ entry clone) and pDestTol2CG2 (destination vector; all vectors from Tol2 kit, (Kwan et al., 2007). Again, correct assembly of the resulting *Tg(mglur6b:EGFP)* vector was checked and confirmed by restriction and partial sequencing.

5.3.3 Transposase mRNA synthesis

pCS2FA-transposase plasmid (Kwan et al., 2007) was linearized with NotI. Capped RNA was transcribed using mMessage mMachine SP6 kit (Ambion), phenol:chloroform extracted and precipitated using isopropanol according to the manufacturer's instructions.

5.3.4 Generation of *Tg(mglur6b:EGFP)zh1* zebrafish

25 pg of *Tg(mglur6b:EGFP)* and 25 pg transposase mRNA were co-injected into one-cell stage zebrafish embryos resulting in EGFP expression in the heart (transgenesis marker expression) and eyes (reporter gene expression). Larvae with EGFP expression in the eyes detectable under a fluorescent Stereomicroscope (Olympus MVX10) were raised to adulthood and outcrossed to wild type (wt) fish. Progeny of 7 *mglur6b:egfp* injected fish were analyzed for transgene expression. Offspring of 3 injected fish had detectable EGFP expression in the eye, with variable rates of transgenesis marker and eye expression between these different founders. F1 larvae showing EGFP expression in the eye were raised to adulthood. Three F1 fish were again outcrossed to wt, all crosses resulting in F2 offspring with clear transgene expression in the eye. Two of these fish, one with strong and one with moderate EGFP expression, were used as founders for *mglur6b:EGFP* transgenic lines. Upon further outcrossing to wt, offspring of the F1 fish with only moderate EGFP expression had lost EGFP expression in the eyes in the F3 generation. However, EGFP expressed in the eyes of F3 offspring of the other F1 fish was clearly visible under a stereomicroscope (Figure 5.2A). This transgenic line was therefore used for all further experiments presented here and is termed *Tg(mglur6b:EGFP)zh1*.

5.3.5 Live observation and live imaging

After gastrulation but before 1 dpf, embryos were treated with 3 µM PTU (1-phenyl-2-thiourea, Sigma-Aldrich) to suppress pigmentation. For observation, larvae were anesthetized with Tricaine (MS-222, Sigma-Aldrich). For live imaging, anesthesized larvae were mounted in 1% low melting temperature agarose (Nu Sieve GTG agarose, Lonza) in E3 medium and covered with E3 medium containing Tricaine. Larvae were observed and imaged using a fluorescent stereomicroscope (Olympus MVX10) equipped with a color camera (ColorViewIII, Soft Imaging System, Olympus).

5.3.6 Whole-mount immunostaining

PTU-treated larvae were fixed in 4% paraformaldehyde for 1 hour at room temperature, dehydrated in an ascending Methanol series (25%, 50%, 75% in PBS and 100%), rehydrated, permeabilized in Acetone (7 minutes at -20°C) followed by two washes in ddH_2O and blocked for 30 minutes at room temperature in PBDT (1% bovine serum albumin, 1% DMSO, 0.1% Triton-X in PBS) supplemented with 10% goat serum. Larvae were incubated in chicken anti-GFP antibody (Table 5.1) diluted in PBDT supplemented with 2% goat serum overnight at 4°C. Secondary antibody (Alexa conjugated goat anti-chicken, 1:500; Molecular Probes, Life Technologies)

Antibody	Immunogen structure	Immunogen structure Manufacturer, catalog number, RRID, species, monoclonal or polyclonal	Concentration
GFP (chicken)	GFP isolated directly from *Aequorea victoria*	Life Technologies, A10262, AB_ 2534023, chicken (IgY), polyclonal	1/500
GFP (rabbit)	E. coli - expressed full length GFP	Torrey Pines Bioloabs, TP401, AB_ 10890443, rabbit, polyclonal	1/500
PKCβ (C-16)	15-25 aa long peptide mapping to the last 50 aa of human PKC βI C-terminus	Santa Cruz Biotechnology, sc-209, AB_ 2168968, rabbit, polyclonal	1/500
ChAT	human placental Choline Acetyltransferase	Millipore, AB144P, AB_ 2079751, goat, polyclonal	1/100
GAD65/67	peptide near C terminus of human GAD65+67 (aa 572-585)	Abcam, Ab11070, AB_ 297722, rabbit, polyclonal	1/200
TH	tyrosine hydroxylase purified from rat PC12 cells	Immunostar, 22941, AB_ 10731005, mouse, monoclonal	1/250

TABLE 5.1: Table of primary antibodies used

was diluted in PBDT and applied for 2 hours at room temperature. Larvae were incubated in Glycerol for at least 1 hour and mounted in Glycerol containing 1.5% low melting temperature agarose (Nu Sieve GTG agarose, Lonza) for imaging.

5.3.7 Immunostaining on sections

Adult zebrafish were euthanized using Tricaine. All tissue was fixed in 4% paraformaldehyde at 4°C. For adult brain sections, the head was cut and fixed overnight. For adult retinal sections, eyes were excised and fixed for 1 hour. Larvae were fixed overnight. Section immunohistochemistry was carried out as described previously (Rinner et al., 2005), except that tissue was embedded in Richard-Allan Scientific Neg-50 Frozen Section Medium (Thermo Fisher Scientific) and cryosectioned at 16 µm thickness. When a primary antibody raised in goat was applied, blocking solution without goat serum was used. For primary antibodies and concentrations, see Table 5.1. Goat, donkey or rabbit anti-chicken, anti-rabbit, anti-mouse or anti-goat IgG conjugated to Alexa 488 or 568 fluorophores (Molecular Probes, Life Technologies), were used at dilutions 1:500 - 1:1000. BODIPY TR Methyl Ester (Molecular Probes, Life Technologies; 1:300) was applied for 20 min after secondary antibodies and nuclei were conterstained with DAPI. Sections were coverslipped with Mowiol mounting medium containing DABCO.

5.3.8 Antibody characterization

Please see Table 5.1 for a list of all primary antibodies used. The PKCβ1 antibody recognized only bands of around 70 kD on Western blots of cell lysates and does not recognize other human PKC isoforms (manufacturer's datasheet). It stained a pattern of cellular morphology and distribution in the zebrafish retina consistent with ON bipolar cell labeling and previous reports (Biehlmaier, Neuhauss, and Kohler, 2003; Yazulla and Studholme, 2001).

The ChAT antibody recognized only a band of around 70 kD on Western blots of mouse brain lysate (see `www.merckmillipore.com` for manufacturer's information), and stained a pattern of cellular morphology and distribution consistent with amacrine cell labeling, identical to previous reports from the teleost retina (Yazulla and Studholme, 2001; Maurer et al., 2010).

The GAD65/67 antibody recognized two bands of expected sizes on Western blots of rat brain lysates (Braden et al., 2010), and stained a pattern of cellular morphology and location in the zebrafish retina consistent with previously used markers for GABAergic amacrine cells in zebrafish (Yazulla and Studholme, 2001).

The TH antibody recognized only a band of around 60 kD on Western blots of cell lysates (manufacturer's datasheet), and stained a pattern of cellular morphology and location in the zebrafish retina identical to previous reports using another TH antibody (Maurer et al., 2010). Double labeling with this second TH antibody (Millipore, AB152, RRID:AB_ 390204) resulted in complete overlap of staining in the retina (not shown).

5.3.9 RNA *in situ* hybridization

A DIG-labeled RNA probe complementary to nucleotides -10 to 1675 of *mglur6b* mRNA was prepared and chromogenic *in situ* hybridization was performed as previously described (Huang et al., 2012) on adult brain cross sections.

5.3.10 Combined fluorescent RNA *in situ* hybridization and immunohistochemistry

Fluorescent RNA *in situ* hybridization was performed as previously described (Huang et al., 2012). For signal development, Alexa Tyramide 488 (TSA Kit# 12, Molecular Probes, Life Technologies) was used. Subsequent to staining for *mglur6b* mRNA, immunohistochemistry using chicken anti-GFP antibody was carried out as described above with the alteration that primary antibody incubation was performed for 1h at RT. As secondary antibody, goat anti-chicken IgG conjugated to Alexa 568 was used. Complete bleaching of transgenically expressed EGFP during the course of *in situ* hybridization was checked and confirmed in a control experiment using Alexa Tyramide 568 instead of Alexa Tyramide 488 and omitting the antibody staining.

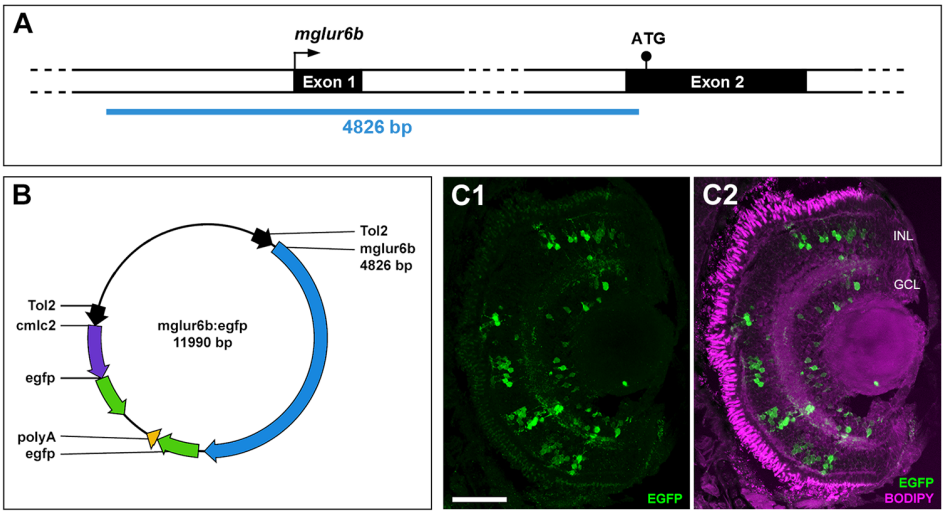


FIGURE 5.1: ***Tg(mglur6b:EGFP)* vector construct and transient EGFP expression.** A 4826 bp long region upstream of the zebrafish *mglur6b* translational start site **(A)** was isolated by PCR and integrated together with EGFP coding sequence and polyA signal in a transgenesis vector containing Tol2 sites and cmlc2:EGFP as transgenesis marker by Multisite Gateway cloning, resulting in Tg(mglur6b:EGFP) **(B)**. Injection of Tg(mglur6b:EGFP) together with transposase mRNA led to EGFP expression in the retinal inner nuclear layer (INL) and ganglion cell layer (GCL) in 5-day-old zebrafish larvae as revealed by immunohistochemistry on larval cross sections **(C)**. Scale bar corresponds to 50 µm. GCL, ganglion cell layer; INL, inner nuclear layer.

5.3.11 Image acquisition and processing

Images of whole mount larvae and overview images of adult brain sections were acquired on a Leica HCS LSI confocal microscope equipped with a 5x zoom objective (Leica Microsystems). Larval sections, adult retinal sections and details of adult brains were imaged on the same Microscope equipped with a 40x oil objective and on a Leica SP5 confocal microscope (Leica Microsystems). Images were adjusted for brightness and contrast using ImageJ (RRID:SCR_ 003070). Figures were composed with Adobe Photoshop CS6 (RRID:SCR_ 014199).

5.4 Results

5.4.1 Retinal transgene expression verifies specificity of *Tg(mglur6b:EGFP)* construct

In order to generate a *Tg(mglur6b:EGFP)* transgenic construct, we cloned a 4.8 kb long upstream fragment of *mglur6b* including the first intron (Figure 5.1A) into a Tol2 transgenesis vector Kwan et al., 2007 that contains a *cmlc2:EGFP* transgenic heart marker for better selection (Figure 5.1B) (for details see Materials and Methods, 76). Around 20% of injected embryos that were positive for the transgenic heart marker showed detectable

EGFP expression in the eye (data not shown). Immunohistochemistry revealed EGFP expression in cells of the inner nuclear layer (INL) and the ganglion cell layer (GCL) (Figure 5.1C), matching the endogenous *mglur6b* expression pattern (Huang et al., 2012). We therefore conclude that the 4.8 kb regulatory region of *mGluR6* contains elements sufficient and necessary for tissue specific *mglur6b* expression. Consequently, we raised a stable transgenic line from injected founders (see Methods, page 77), termed *Tg(mglur6b:EGFP)zh1*.

5.4.2 EGFP expression pattern in the developing central nervous system suggests yet unidentified *mglur6b* expression domains

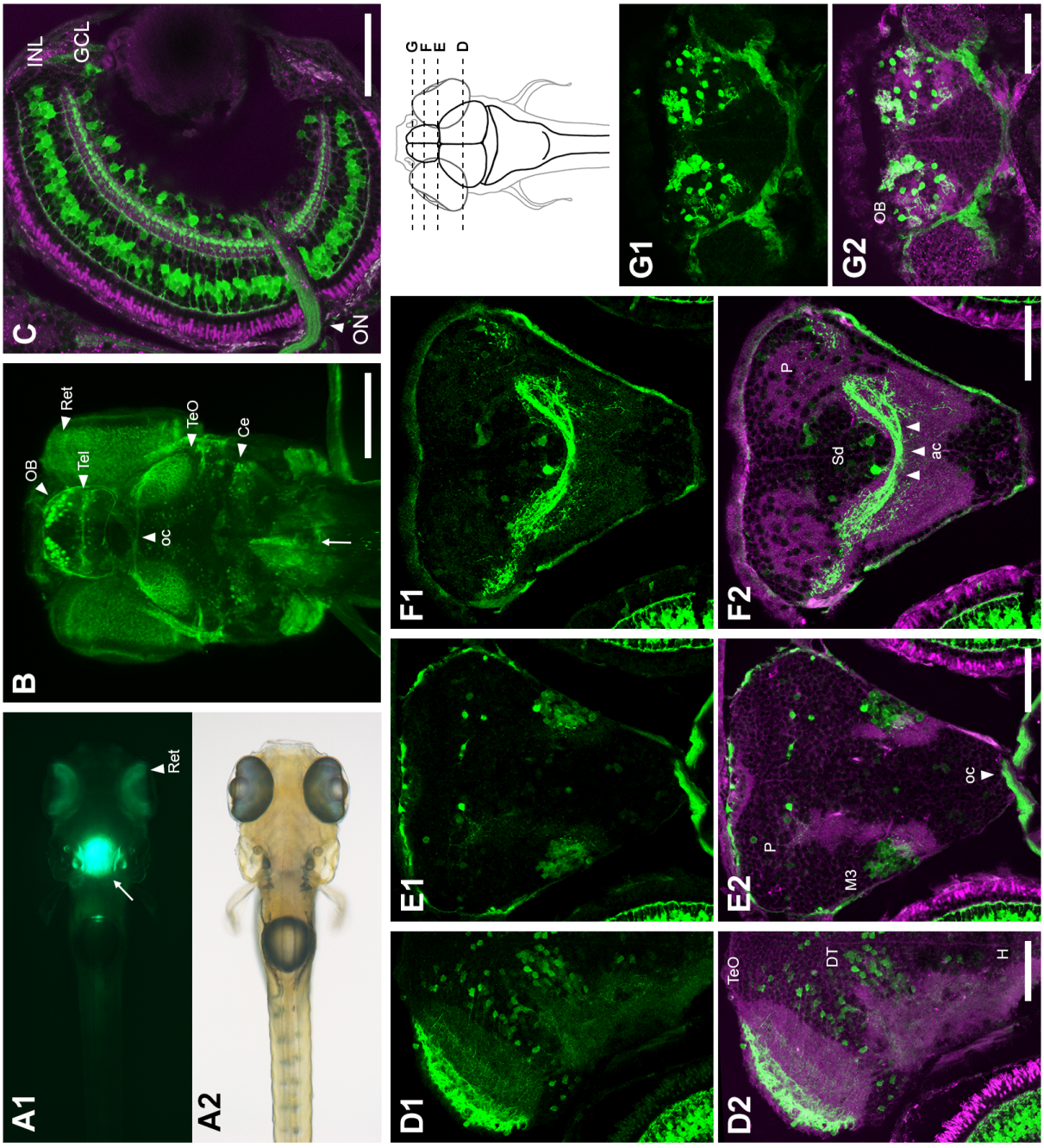
EGFP expression in the eyes of *Tg(mglur6b:EGFP)zh1* larvae can be observed from 3 dpf onwards (Figure 5.2A,B). Histological sections reveal EGFP positive cells in the INL and GCL (Figure 5.2C), just as in *Tg(mglur6b:EGFP)* injected fish (Figure 5.1C). Based on morphology and location of EGFP positive cell bodies in the INL, we conclude that a subset of bipolar and amacrine cells are labeled. In addition, a subpopulation of ganglion cells expresses EGFP, evident by partial labeling of the optic nerve (Figure 5.2C). We cannot exclude however that at least some of the labeled cells in the GCL are displaced amacrine cells.

Additional EGFP expression in a number of brain regions (Figure 5.2B, D, E) becomes evident by confocal microscopy. For identification of labelled brain regions and their nomenclature, we generally referred to the zebrafish brain atlas of larval (Mueller and Wullimann, 2005) and adult (Wullimann, Rupp, and Reichert, 1996) zebrafish. In the optic tectum, not only terminals of retinal ganglion cells are EGFP positive, but also cell bodies within the optic tectum (Figure 5.2B,D) are weakly labeled. Furthermore, EGFP positive cells are present in the cerebellum (Figure 5.2B), dorsal thalamus (Figure 5.2D), migrated area of eminentia thalami (Figure 5.2E) and the hypothalamus (Figure 5.2D). In the telencephalon, both pallium and subpallium contain labelled cells (Figure 5.2E, F). However, by far the strongest signal can be seen in a subset of olfactory bulb neurons and projectons to the habenula (Figure 5.2G) (Miyasaka et al., 2009), indicating specific transgene expression in olfactory bulb projection neurons. These findings suggest the discovery of yet unidentified *mglur6b* expression domains.

5.4.3 EGFP expression in the brain is maintained up to adulthood

We next examined transgene expression in the adult brain. Similar to larvae, EGFP expression was found in optic tectum, cerebellum, thalamic region, hypothalamus, pallium, subpallium and most prominently in the olfactory bulb. Thus, expression in *Tg(mglur6b:EGFP)zh1* appears to remain largely unchanged after 5 dpf.

For our following analysis, we focus on selected brain regions which show strong or cell type specific expression. Our approach is particularly suited for cell-type specific *mglur6b* expression analysis, since transgenically expressed EGFP labels entire neurons including their processes, yielding information about their shape and connectivity.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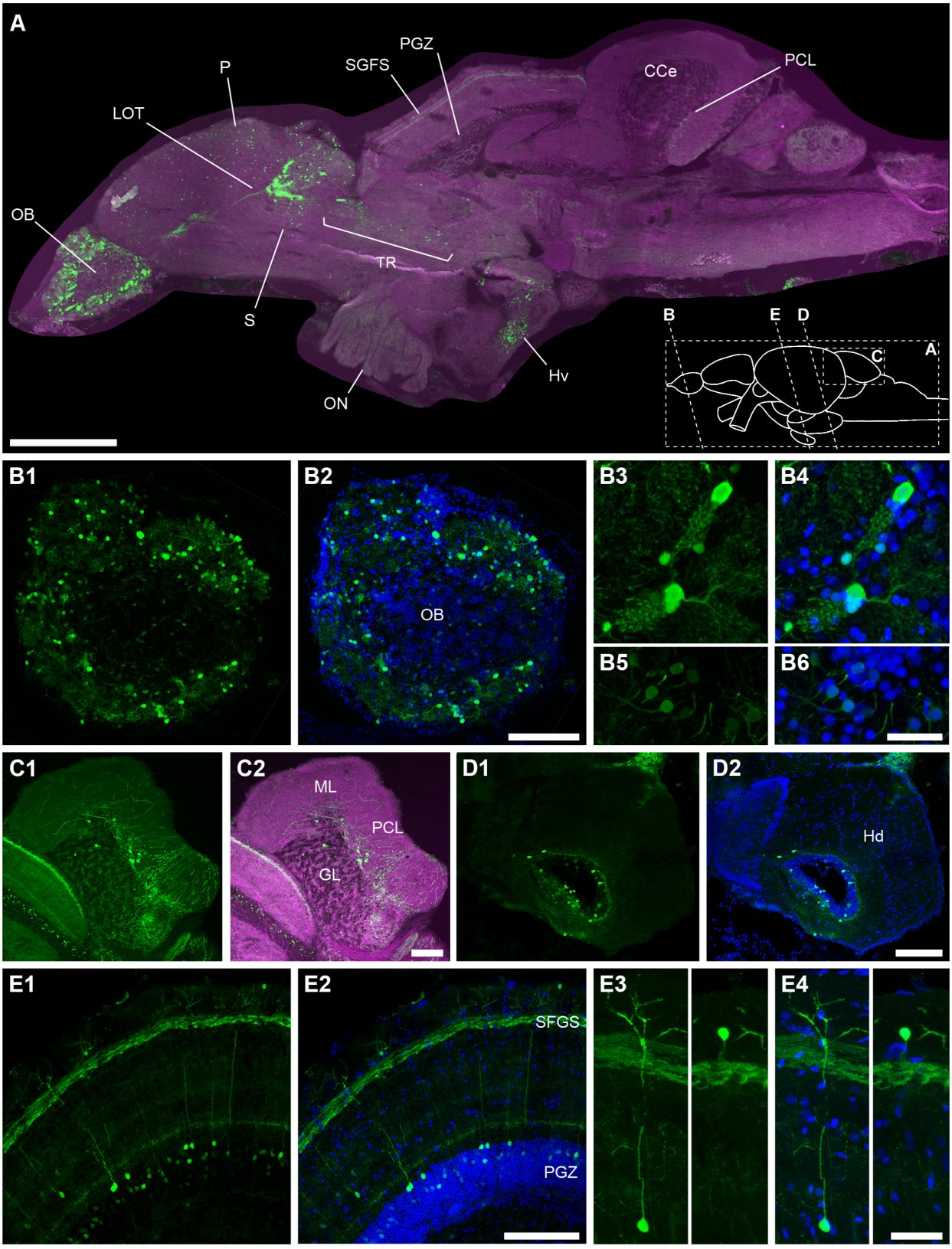
5.4.4 Olfactory bulb projection neurons express *mglur6b*

In *Tg(mglur6b:EGFP)zh1*, the olfactory bulb shows strong EGFP expression in its outer region, whereas the central area is largely devoid of signal, with the exception of few weakly labeled cells, likely being olfactory bulb interneurons (Figure 5.3A,B). The outer region of the zebrafish olfactory bulb is mainly populated by mitral and ruffed cells, constituting the olfactory bulb projections neurons. (Byrd and Brunjes, 1995; Fuller and Byrd, 2005; Fuller, Yettaw, and Byrd, 2006; Rink and Wullimann, 2004). The typical ruffed cell morphology, characterized by elaborate membrane protrusions (ruffs) at the initial segment of the axon, can easily be observed in EGFP expressing cells (Figure 5.3B3,4). Ruffs can be found on many, but not all labelled cells in the olfactory bulb outer regions. Therefore, very likely mitral cells also express EGFP (Figure 5.3B5,6). Since mitral and ruffed cells are the projection neurons of the olfactory bulb, the lateral olfactory tract (Figure 5.3A) is also labeled, confirming labeling of olfactory bulb output neurons. Fluorescent RNA *in situ* hybridization using an *mglur6b* antisense probe in combination with antibody staining for EGFP shows that *mglur6b* is indeed strongly expressed in ruffed and probably also mitral cells (Figure 5.4A).This suggests that *mGlur6b* may play a role in olfactory processing, at least on the level of olfactory bulb output neurons.

FIGURE 5.2 *(preceding page)*: **EGFP expression in 5-day-old *Tg(mglur6b:EGFP)zh1* zebrafish.** Live imaging **(A)**, whole-mount immunohistochemistry **(B)** and immunohistochemistry on cross sections of 5-day-old zebrafish **(C-G)**. Wide-field live observation (**A)** shows EGFP expression in the retina. Green fluorescence of the heart **(A,B**, arrows**)** is due to expression of the *cmlc2:EGFP* transgenesis marker contained in the transgenesis vector. Whole-mount immunohistochemistry followed by confocal microscopy (**B)** reveals additional EGFP expression in cell bodies of the olfactory bulb (OB), a small number of other telencephalic neurons (Tel), the optic tectum (TeO) and the cerebellum (Ce). Note that also optic nerves (ONs) emerging from retinal ganglion cells **(C)**, crossing in the optic chiasm (oc) **(B,E)** and projecting to the TeO **(B,D)** are labeled. Within the retina, EGFP is expressed in the INL and GCL. Cross sections through the brain imaged with confocal microscopy reveal EGFP in cell bodies of the TeO **(D)**, dorsal thalamus (DT) **(D)**, hypothalamus **(D)**, pallium (P) **(E,F)**, migrated area of eminentia thalami (M3) **(E)**, dorsal division of subpallium (Sd) **(F)** and OB **(G)**. Green, EGFP; Magenta, BODIPY. Scale bars correspond to 200 µm (B) and 50 µm **(C-E)**. ac, anterior commissure; Ce, cerebellum; DT, dorsal thalamus; GCL, ganglion cell layer; EmT, eminentia thalami; H, hypothalamus; INL, inner nuclear layer; M3, migrated area of eminentia thalami; OB, olfactory bulb; oc, optic chiasm; ON, optic nerve; P, pallium; Ret, retina; Sd, dorsal division of subpallium; Tel, telencephalic neurons; TeO, optic tectum.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B6
C1
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5.4.5 *Mglur6b* is expressed in distinct cell types of the optic tectum

The optic tectum of teleost fishes is a major projection area for retinal ganglion cells, integrating and processing sensory information. The laminar structure of the optic tectum harbors a deep cell body layer (periventricular grey zone (PGZ)) and a superficial neuropil layer containing axons and dendrites from cells of PGZ (Nevin et al., 2010). The majority of EGFP expressing cell bodies within the optic tectum is located in the PGZ (Figure 5.3E). A few EGFP positive cell bodies can additionally be found within the neuropil layer. In some of them, heavily branched projections to the outermost layer of the optic tectum can be observed (Figure 5.3E), reminiscent of pyramidal cells known from the optic tectum of other teleost fishes (Vanegas and Ito, 1983; Meek and Schellart, 1978). We also found EGFP expression in cell bodies residing distally to the major projection lamina of retinal ganglion cells (stratum fibrosum et griseum superficiale), representing superficial interneurons (Nevin et al., 2010). Since the optic tectum contains a population of cholinergic cells (Mueller, Vernier, and Wullimann, 2004), we checked whether EGFP expressing cells are also positive for acetylcholinesterase (ChAT). However, both in larvae and adults, EGFP and ChAT label non-overlapping subpopulations of tectal neurons (Figure 5.5). RNA *in situ* hybridization shows that not only EGFP, but also *mglur6b* is expressed in the adult optic tectum (Figure 5.4C). These findings suggest a more widespread role of mGluR6 in the visual pathway than previously described, extending mGluR6 function from retinal signaling to signaling

FIGURE 5.3 *(preceding page)*: **EGFP expression in adult *Tg(mglur6b:EGFP)zh1* zebrafish brain.** Confocal images of sagittal **(A,C)** and cross sections **(B,D,E)** of adult zebrafish brain stained with EGFP antibody. The olfactory bulb (OB) is strongly labeled in its outer region **(A,B)**, and higher magnification reveals strong labeling of mitral/ruffed cells with their typical morphology, whereas some other OB neurons (granule cells or interneurons) express EGFP weakly in comparison **(B)**. Few cell bodies in the Corpus cerebelli (CCe) **(C)** are EGFP positive, resembling Purkinje cells due to their neurites extending into the molecular layer. EGFP positive cells can also be seen in the ventral Hv **(A)** and dorsal **(D)** periventricular hypothalamus (Hd), as well as in pallium (P)) and subpallium (S), and the thalamic region (TR) **(A)**. Within the optic tectum (TeO), EGFP expressing cell bodies reside in the periventricular grey zone (PGZ), within the neuropil layer (arrows) and close to the optic tectum surface (superficial interneurons, arrowheads) **(E)**. Green, EGFP; magenta, BODIPY; blue, DAPI. Scale bars correspond to 500 µm (A), 100 µm **(B′, C′, D′. E′)**, 50 µm **(E″′)** and 25 µm **(B″′)**. CCe, Corpus cerebelli; GL, granule cell layer of CCe; Hd, periventricular hypothalamus; Hv, ventral hypothalamus; LOT, lateral olfactory tract; ML, molecular layer of CCe; OB, olfactory bulb; ON, optic nerve; P, pallium; PCL, purkinje cell layer; PGZ, periventricular grey zone; S, subpallium; SFGS, superficial grey/fibrous layer of optic tectum; TeO, optic tectum; TR, thalamic region.

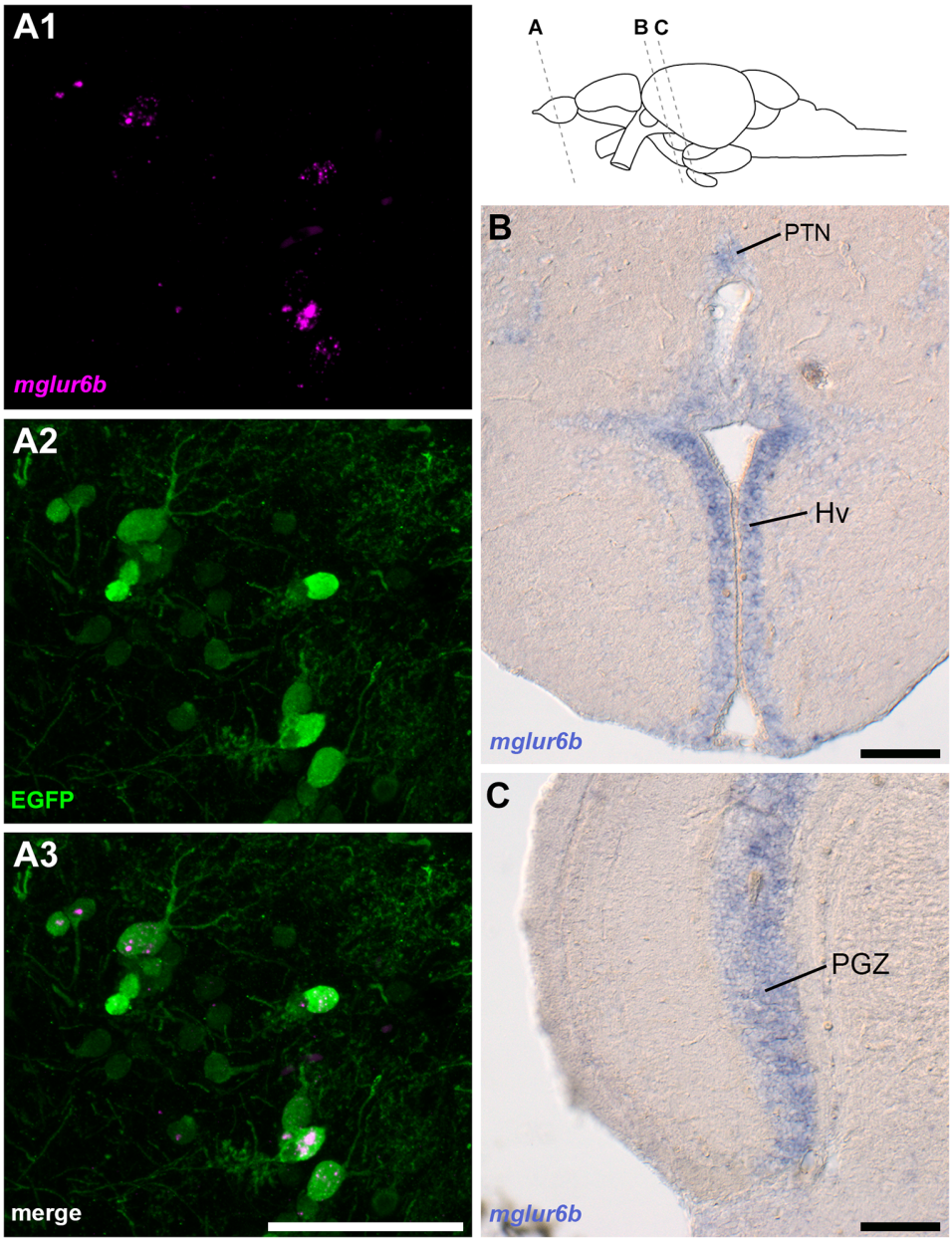

FIGURE 5.4: ***mglur6b* mRNA expression in the adult zebrafish brain.** Confocal images of fluorescent *in situ* hybridization combined with EGFP antibody staining on cross sections of *Tg(mglur6b:EGFP)zh1* olfactory bulb **(A)** and bright field microscopy of chromogenic *in situ* hybridization on cross sections of wild type (wt) ventral hypothalamus (Hv) (B) and optic tectum (TeO) **(C)**. In the olfactory bulb **(A)**, both *mglur6b* mRNA (magenta) and EGFP (green) expression is found in ruffed cells (arrows) and very likely also in mitral cells (arrowheads). In Hv **(B)**, *mglur6b* is detected in the periventricular zone. Also the posterior tuberal nucleus (PTN) expresses *mglur6b* **(B)**. In TeO **(C)**, *mglur6b* is clearly detected in the periventricular grey zone (PGZ), whereas expression in single cell bodies within the neuropil layer remains questionable. Scale bars correspond to 50 µm. Hv, ventral hypothalamus; PGZ, periventricular grey zone; PTN, posterior tuberal nucleus.

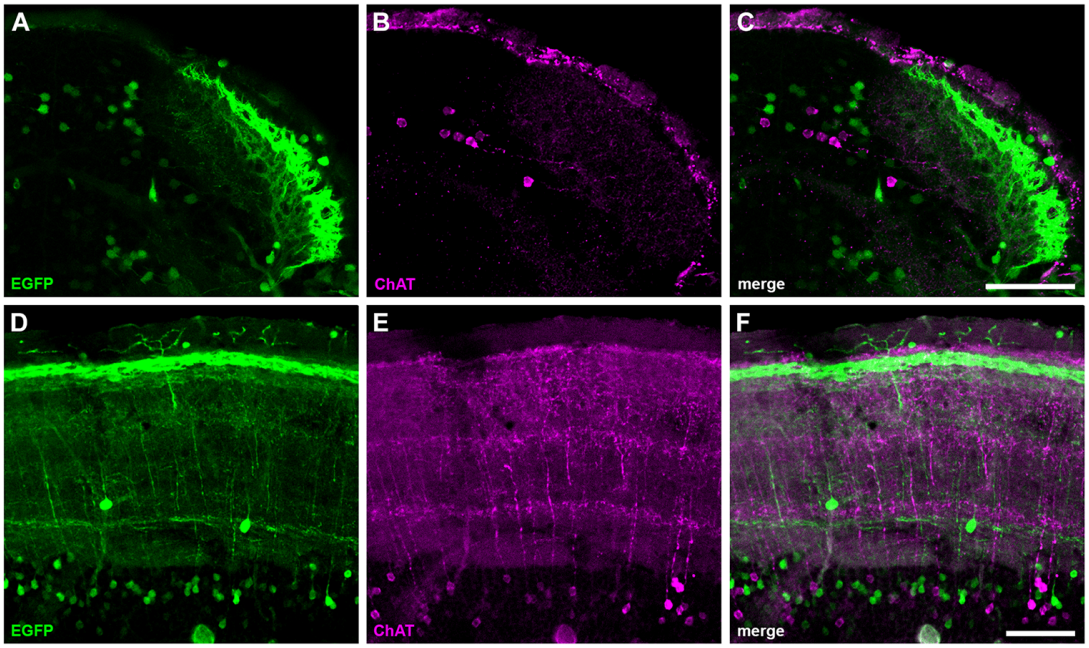

FIGURE 5.5: **Analysis of EGFP expressing cells in the optic tectum.** Double labeling of EGFP (green) and ChAT (magenta) demonstrates non-overlapping *mglur6b:EGFP* expressing and cholinergic subpopulations of TeO neurons in 5-day-old (A-C) and adult (D-F) zebrafish. Scale bars correspond to 50 µm.

in the major visual processing center of the brain.

5.4.6 Labeling of ON and OFF bipolar cell subtypes in *Tg(mglur6b:EGPF)zh1*

EGFP expression in the adult retina is found in the same layers as in larvae, consistent with the previously published adult retinal expression pattern of *mglur6b* (Huang et al., 2012). *Tg(mglur6b:EGFP)zh1* shows labeling of bipolar cells and amacrine or displaced ganglion cells within the INL, and ganglion cells and potentially displaced amacrine cells in the GCL (Figure 5.6).

In the central INL of the retina, numerous cells with typical bipolar cell morphology are labeled. Double-labeling with PKCβ1, a marker for ON bipolar cells, shows substantial overlap of EGFP and PKCβ1 positive cells (Figure 5.7A-D). However, not all ON bipolar cells are labeled in *Tg(mglur6b:EGPF)zh1*. Roughly one sixth of PKCβ1 positive cells are negative for EGFP. The vast majority of these cells show the typical morphology of the s6L bipolar cell with its big axon terminal in the proximal IPL (Connaughton, Graham, and Nelson, 2004). Conversely, more than 1/3 of EGFP positive cells do not co-label with PKCβ1 and are located distally to all PKCβ1 positive terminals (Figure 5.7A-C), putting into question the expectedly exclusive transgene expression in bipolar cells of the ON type. Indeed, close examination of the IPL shows that there are bipolar cell boutons labeled in the distal half of the IPL (Figure 5.7E-H), generally considered

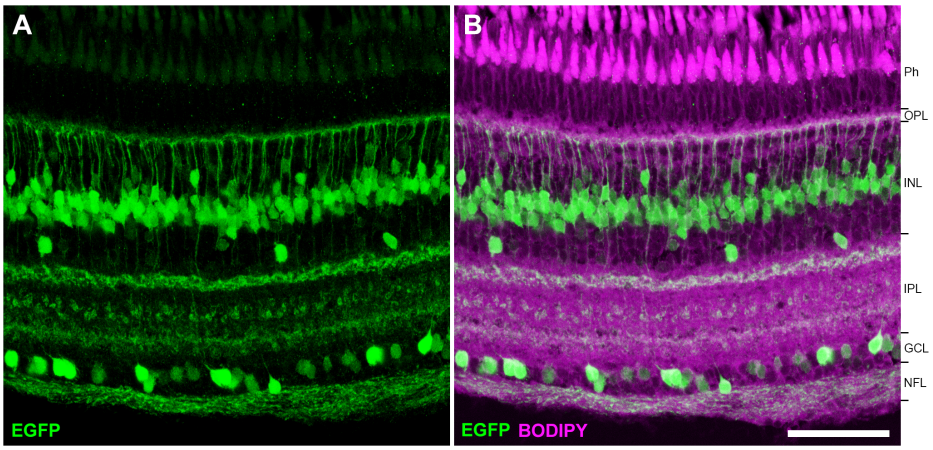

FIGURE 5.6: **EGFP expression in adult *Tg(mglur6b:EGFP)zh1* zebrafish retina.** Confocal image of a retinal section stained with EGFP antibody (green) **(A)** and overlay with BODIPY (magenta) **(B)**. EGFP expressing cells within the inner nuclear layer (INL) display morphology and location of bipolar and amacrine or displaced ganglion cells. In the ganglion cell layer (GCL), retinal ganglion cells are EGFP positive, evidenced by labeling of the optic nerve fiber layer. Scale bar corresponds to 50 µm. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NFL, nerve fiber layer; OPL, outer plexiform layer Ph, photoreceptor layer.

the OFF sublamina. All known mixed ON/OFF bipolar cell types (Connaughton, Graham, and Nelson, 2004) have axon terminals in sublaminae devoid of EGFP positive bipolar cell terminals. Taken together, these findings indicate labeling of OFF bipolar cells in *Tg(mglur6b:EGPF)zh1*.

5.4.7 *Tg(mglur6b:EGPF)zh1* reveals a novel amacrine cell type

To further elucidate the identity of labeled cells in the proximal INL of the retina, we performed immunohistochemistry for known markers of amacrine cells. EGFP positive cells in the proximal INL are all positive for ChAT, a marker for cholinergic neurons (Figure 5.8A-C). ChAT is prominently expressed in starburst amacrine cells across diverse species (Masland and Tauchi, 1986; Vaney, 1990; Yazulla and Studholme, 2001). Absence of overlapping EGFP and ChAT immunoreactivity (Figure 5.8A-C) in the GCL argues however against transgene expression in starburst amacrine cells, which typically show a mirror-like arrangement in INL and GCL. Moreover, unlike starburst amacrine cells, the EGFP expressing cells in *Tg(mglur6b:EGFP)zh1* are not GABAergic, as revealed by staining for GAD65/67 (Figure 5.8D-F). Due to the low number of EGFP positive cells in the proximal INL we suspected that labeled cell might be dopaminergic. However, tyrosine hydroxylase immunoreactivity, marking dopaminergic neurons, does not overlap with EGFP staining (Figure 5.8G-I) suggesting that these EGFP positive cells represent an amacrine cell type described for the first time in teleost fishes.

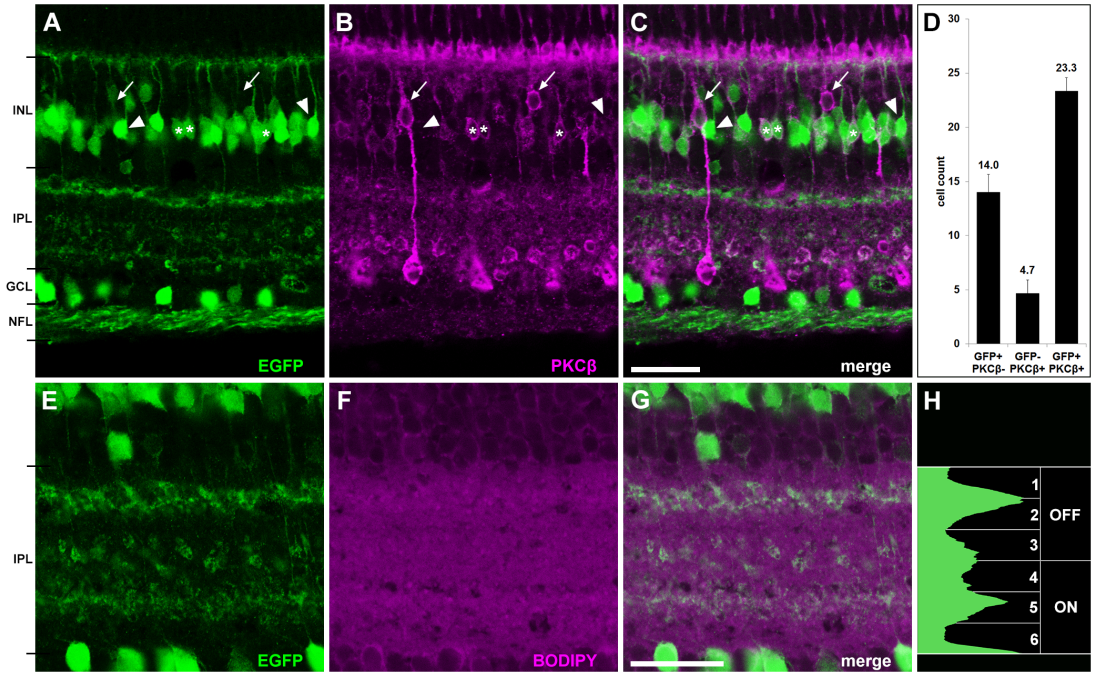

FIGURE 5.7: **Analysis of EGFP expressing retinal bipolar cells.** Double labeling of EGFP and ON bipolar cell marker PKCβ **(A-D)** and labeling of EGFP counterstained with BODIPY with focus on the inner plexiform layer (IPL) **(E-H)**. EGFP (green) and PKCβ (magenta) positive cells are substantially overlapping. EGFP positive / PKCβ negative cells and EGFP negative / PKCβ positive cells are also evident **(A-C)**. Note the absence of EGFP in s6L bipolar cells strongly labeled by PKCβ. The experiment was quantified by counting labeled cells in retinal stretches of 150 µm in retinal sections from 3 adult *Tg(mglur6b:EGFP)zh1* zebrafish **(D)**. The entire thickness of the IPL was visualized by staining with BODIPY (magenta) **(F,G)**, and subdivided into 6 sublayers of equal thickness according to Connaughton, Graham, and Nelson, (2004) **(H)**. Fluorescence intensity was quantified as mean intensity value across the inner plexiform layer using ImageJ **(H)**. Axon terminals of EGFP positive bipolar cells (green) with typical bouton-like morphology can be identified in sublayers 3, 4 and 5 of IPL **(E-G)**. EGFP signal at the border between sublayers 1 and 2 is more diffuse and probably represents neurites from other labeled cell types (ganglion cells, amacrine cells), although small bipolar cell axon terminals may also be contained **(E-G)**. Scale bars correspond to 25 µm. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NFL, nerve fiber layer.

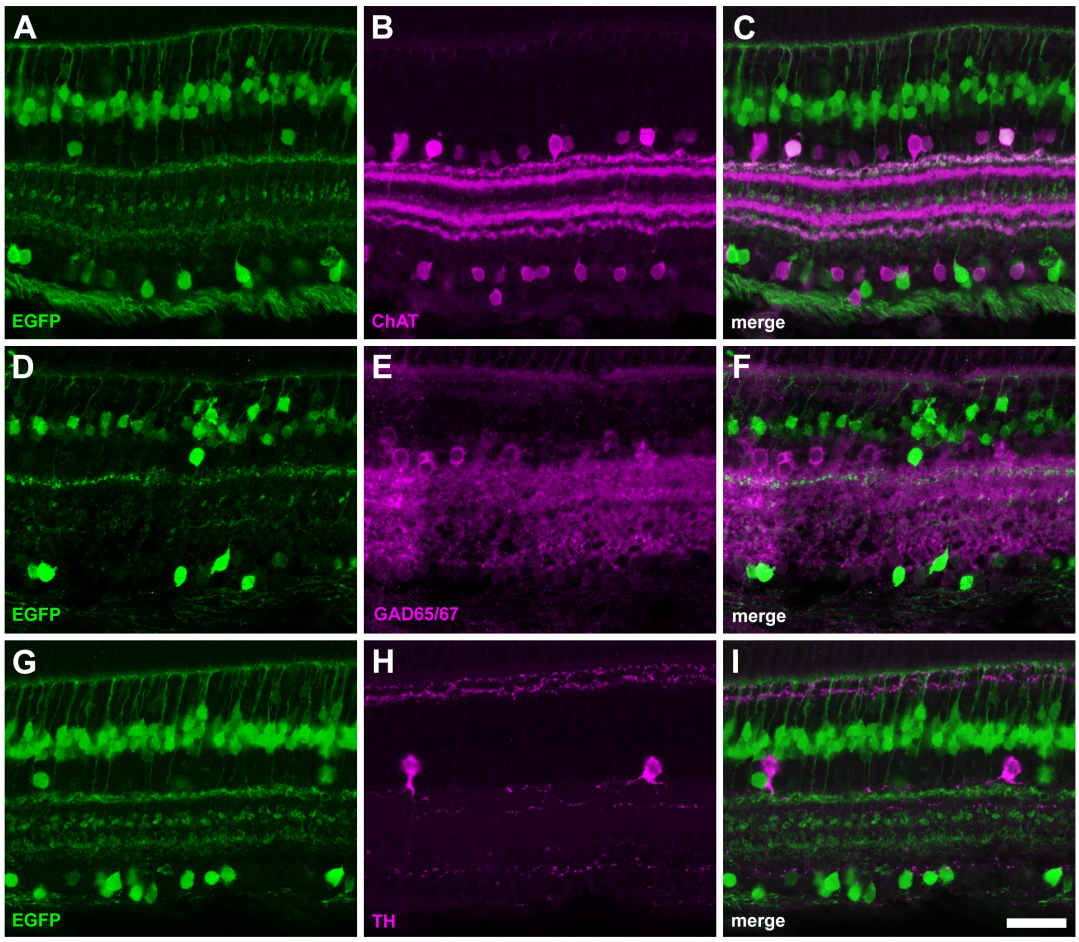

FIGURE 5.8: **Analysis of EGFP expressing amacrine cells.** Double labelings of EGFP with cholinergic cell marker acetylcholinesterase **(A-C)**, GABAergic cell marker GAD65/67 **(D-F)** and dopaminergic cell marker tyrosine hydroxylase **(G-I)**. All EGFP positive cells (green) with morphology and location of amacrine cells overlap with acetylcholinesterase (magenta) immunoreactivity. Acetylcholinesterase and EGFP staining do not overlap in the ganglion cell layer **(A-C)**. There is no overlap between EGFP (green) and GAD65/67 (magenta) positive cell bodies **(D-F)** or tyrosine hydroxylase (magenta) positive cell bodies **(G-I)**. Scale bar corresponds to 25 µm.

5.5 Discussion

In this study, we generated a new transgenic zebrafish line, *Tg(mglur6b:EGFP)zh1*, in order to identify brain regions and neuronal cell types using mGluR6b to sense glutamate. We employed live imaging and immunohistochemistry to visualize EGFP expressing cells and RNA *in situ* hybridization to detect *mglur6b* transcripts. mGluR6 is so far only known for its role in sign inversion at the ON bipolar cell synapse, and expression is generally considered to be restricted to ON bipolar cells. Therefore, it seems surprising that transgene as well as *mglur6b* mRNA expression (this study and Haug et al., 2013 are found in a number of other retinal cell types and specific brain regions, including olfactory bulb and optic tectum. Our findings open the exciting possibility that mGluR6 mediated sign inversion plays a role at other synapses besides the photoreceptor-ON bipolar cell synapse, which would imply that glutamatergic inhibition in the vertebrate central nervous system is more common than currently appreciated.

5.5.1 EGFP expression recapitulates mglur6b mRNA expression

Within the *Tg(mglur6b:EGFP)zh1* retina, subpopulations of bipolar, amacrine and ganglion cells are EGFP positive. In addition, EGFP is expressed in cell bodies within optic tectum, hypothalamus, cerebellum, thalamus, olfactory bulb and in some cells of the remaining telencephalon. These regions were labeled in larval as well as adult zebrafish, and transgene expression is largely consistent with previously published *mglur6b* expression patterns in larval zebrafish (Haug et al., 2013; Huang et al., 2012). However, so far *mglur6b* expression has not been reported from optic tectum, thalamus and cerebellum, and expression has not yet been analyzed in adult brain. Here, we were able to confirm *mglur6b* expression in the adult olfactory bulb (Figure 5.4A), hypothalamus (Figure 5.4B) and optic tectum (Figure 5.4C) by RNA *in situ* hybridization. The fact that transgene and *mglur6b* mRNA expression in the optic tectum are weak compared to other expression domains likely accounts for not having detected it previously in larvae.

Interestingly the habenula has been shown to be weakly *mglur6b* mRNA positive (Haug et al., 2013), but the transgene is absent in habenular cell bodies. As expression in the olfactory bulb is very strong, and trafficking of mRNAs coding for synaptic proteins is known to occur (Zivraj et al., 2010; Taylor et al., 2009), this discrepancy might be explained by the fact that *mglur6b* mRNA could be located in axons of olfactory bulb neurons rather than habenular cell bodies. Double labeling of *mglur6b* mRNA and EGFP directly demonstrated transgene and *mglur6b* mRNA expression in the same olfactory bulb neurons.

5.5.2 Multiple roles of mGluR6b in visual signal processing

EGFP and *mglur6b* mRNA are expressed in multiple cell types contributing to visual signal processing: Within the retina, where visual information is perceived, filtered and further processed, transgene expression is evident in subtypes of bipolar, amacrine and ganglion cells. Within the optic tectum, constituting the major brain area for integrating visual and other sensory information in teleost fishes, several cell types displaying distinct locations and morphologies are labeled. Transgene expression in tectal superficial

interneurons is intriguing, since these cells have been proven necessary for size filtering (Del Bene et al., 2010), and it would be interesting to see whether mGluR6b contributes to this function.

In line with reported expression and function of mGluR6 across vertebrates, double labeling with PKCβ confirmed transgene expression in ON bipolar cells. The existence of PKCβ / EGFP negative cells demonstrates that transgene expression is restricted to a subpopulation of ON bipolar cells. PKCβ positive / EGFP negative bipolar cells constitute one specific bipolar cell type, the Bon-s6L cell (Connaughton, Graham, and Nelson, 2004) , which can easily be identified by strong PKCβ labeling, a big cell body and a big axon terminal in the proximal INL. This cell type is known to receive predominant input from rods (Li et al., 2012). Interestingly, it has been previously suggested that the rod pathway relies more on the receptor encoded by the *mglur6a* paralogue, while *mglur6b* is more important for the cone ON pathway (Haug et al., 2013). Our finding supports this hypothesis.

Unexpectedly, we also detected EGPF positive / PKCβ negative cell bodies and EGFP positive bipolar cell terminals within the inner plexiform OFF sublayer, two findings suggesting transgene expression in OFF bipolar cells. Due to the high degree of overlap between *mglur6b* and EGFP expression in *Tg(mglur6b:EGFP)zh1*, we consider this a very likely possibility. Alternatively, EGFP positive axon terminals within the upper half of the inner plexiform layer may actually belong to ON bipolar cells, as they are located in proximity to the ON sublayer. This would however imply that the subdivision of the inner plexiform layer generally agreed on in zebrafish (Connaughton, Graham, and Nelson, 2004) is not completely accurate. Electrophysiological examination would be needed to resolve whether the transgene is indeed expressed in OFF bipolar cells. If mglur6b is expressed in OFF bipolar cells as suggested, we expect it to have a role other than sign inversion at the photoreceptor synapse, implying that mGluR6 may not be entirely specialized on glutamatergic inhibition.

Interestingly, transgene expression also reinforces the surprising finding of *mglur6b* expression in retinal ganglion cells (Huang et al., 2012). Despite a strong tenet of mGluR6 expression being restricted to bipolar cells, there is literature suggesting mGluR6 expression in retinal ganglion cells even outside teleost fishes (Tehrani, Wheeler-Schilling, and Guenther, 2000; Morgan et al., 2006; Klooster et al., 2011). In murine ganglion cells, mGluR6 was only detected during development and after injury (Tehrani, Wheeler-Schilling, and Guenther, 2000), pointing to a role in development or plasticity. The situation might be different in zebrafish, as expression in retinal ganglion cells is maintained up to adulthood, arguing rather for a role in synaptic modulation than in development.

5.5.3 mGluR6b signaling in olfactory bulb and other brain areas

EGFP and mglur6b mRNA are strongly expressed in projection neurons of the olfactory bulb, raising the exciting possibility that mGluR6b has a hyperpolarizing role in mitral/ruffed cell inhibitory coupling, similar to its established function in the photoreceptor-ON bipolar cell synapse. Interestingly, transgene expression has been previously reported in accessory olfactory bulb mitral cells of mGluR6 transgenic mice (Vardi et al., 2011). Although this study did not directly address endogenous mGluR6 expression,

together with our findings it suggests evolutionary conserved mGluR6 signaling in the olfactory bulb. However, it will require physiological studies to see whether mitral/ruffed cells receive inhibitory glutamatergic input similar to retinal ON bipolar cells.

Within the cerebellum, EGFP is expressed in the Purkinje cell layer, characterized by its location between granule cell and molecular layer. Since endogenous mRNA expression has not been reported from the cerebellum, it remains to be determined whether mGluR6b plays a role in cerebellar signaling. Both the EGFP transgene and *mglur6b* mRNA are also expressed in the hypothalamus (Haug et al., 2013). To our knowledge, the hypothalamus is the only brain region apart from the retina that clearly has been reported to express MGLUR6 in mammals (Ghosh, Baskaran, and van den Pol, A N, 1997). This conserved expression points to an important role of mGluR6 in hypothalamic function.

5.5.4 Discovery of a novel amacrine cell type by *Tg(mglur6b:EGFP)zh1*

In the zebrafish retina, 28 morphologically distinct amacrine cell types have been described so far (Jusuf and Harris, 2009). Like other vertebrates, zebrafish possess cholinergic amacrine cells (Yazulla and Studholme, 2001). Cholinergic amacrine cells have been generally termed starburst amacrine cells due to their morphology, and they have been shown to be essential for direction selectivity of retinal ganglion cells. GABA, which is also released by starburst amacrine cells, is important for conveying this direction selectivity (Taylor and Smith, 2012; Caldwell, Daw, and Wyatt, 1978). To our surprise, we found transgene expression in cholinergic, non GABAergic amacrine cells that also do not display typical mirror-like arrangement across the inner plexiform layer. Therefore, *Tg(mglur6b:EGFP)zh1* labels an amacrine cell type not previously described in teleost fishes. While starburst amacrine cells are often considered the only cholinergic retinal cells, there is a number of reports demonstrating cholinergic cells of non-starburst type in mammalian and non-mammalian retinae (Guiloff and Kolb, 1992; Millar et al., 1985; Schmidt, Wassle, and Humphrey, 1985; Conley, Fitzpatrick, and Lachica, 1986; Sandmann, Engelmann, and Peichl, 1997). Most similar to our finding, cholinergic, non-GABAergic amacrine cells lacking typical starburst cell arrangement were found in the proximal INL of the ground squirrel retina (Cuenca et al., 2003). Since the role of cholinergic synapses in retinal signaling is still poorly understood, the function of this cell type is currently unknown.

5.5.5 Conclusion

We have generated a new transgenic zebrafish line, *Tg(mglur6b:EGFP)zh1*, well recapitulating endogenous *mglur6b* expression. Expression of the transgene as well as *mglur6b* in cells besides the previously described ON bipolar cells strongly argues for additional function of this receptor besides its role at the photoreceptor-ON bipolar cell synapse. In particular, our data extend mGluR6b-mediated glutamate signaling to multiple levels of visual signal processing including the optic tectum, and to other brain areas, most prominently the olfactory bulb.

It is very likely that such additional functions also exist outside of teleost fishes, as indicated by a number of reports (Tehrani, Wheeler-Schilling, and Guenther, 2000; Dhingra et al., 2008; Klooster et al., 2011; Vardi et al., 2011; Ghosh, Baskaran, and van den Pol, A N, 1997). Which specific role mGluR6b plays in these other neuronal cell types remains an open question. Two mutually non-exclusive possibilities seem most likely: First, mGluR6b might have a neuromodulatory role, similar to reported function of other group III mGluRs. Second, mGluR6b might mediate synaptic transmission with glutamate acting as inhibitory neurotransmitter, similar to its role in ON bipolar cells. Although glutamate is generally considered an excitatory neurotransmitter, there are reports showing cases of glutamatergic inhibition in both invertebrates (Chalasani et al., 2007; Liu and Wilson, 2013) and vertebrates (Lee and Sherman, 2009; Cox and Sherman, 1999; Sekizawa et al., 2009), and mGluRs of group II and III have been shown to be responsible for these effects in vertebrates.

To shed more light on novel mGluR6 functions, it will be important to establish subcellular localization of mGluR6b protein, since postsynaptic localization would be in favor of a role in direct synaptic transmission. Further, physiological studies of EGFP expressing cells in *Tg(mglur6b:EGFP)zh1* will give us more insight into their function and the function of mGluR6b. This would be particularly interesting in combination with pharmacological inhibition of mGluRs or genetic knockout of *mglur6b*, which, with the establishment of CRISPR/Cas9 system (Hwang et al., 2013), is now readily possible in zebrafish.

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5.7 Conflict of interest

The authors declare no conflict of interest.

5.8 Author contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. SMGK: study concept and design, establishing transgenic line, data acquisition and analysis, drafting, writing and revision of the manuscript; RW: establishing transgenic line, revision of the manuscript; MG: study design, revision of the manuscript; SCFN: study supervision, concept and design, drafting and revision of the manuscript.

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Chapter 6

Zebrafish as Model for Cone Photoreceptor Dysfunction: Investigating the Role of Ca^{2+} Channel $\alpha_2\delta_4$ Subunits in the Retina

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6.1 Abstract

Photoreceptors rely on specialized ribbon synapses to transmit graded and sustained light responses over a broad range of light intensities. Tonic release of synaptic vesicles from photoreceptors is mediated by high-voltage activated Ca^{2+} channels, consisting of pore-forming α_1 and auxiliary $\alpha_2\delta$ and β subunits. $\alpha_2\delta$ subunits are extracellular, GPI anchored proteins shown to be important for targeting of Ca^{2+} channel complexes to synaptic compartments of the plasma membrane. In the human genome, four genes encoding $\alpha_2\delta$ subunits, *CACNA2D1* – *CACNA2D4*, exist. Mutations in *CACNA2D4* have been found to cause cone photoreceptor dysfunctions in human patients. In order to gain insight into mechanisms leading to onset and of progression of these retinal defects, we investigated expression and function of *cacna2d4* genes in the cone-dominant zebrafish retina.

The zebrafish genome harbors two orthologous genes to human *CACNA2D4*, termed *cacna2d4a* and *cacna2d4b*. Both *cacna2d4a* and *cacna2d4b* show specific expression in photoreceptors and cells of the inner nuclear layer during development and adulthood, indicating conserved expression between mammals and zebrafish. In order to study loss of $\alpha_2\delta_4$ function, we generated *cacna2d4a* and *cacna2d4b* mutant zebrafish lines employing the CRISPR/Cas9 system. Mutagenesis was highly efficient, and frameshift mutations were successfully transmitted through the germ line. Mutation of *cacna2d4b* causes mislocalization of Cacna1fa, the principle $\alpha 1$ subunit in photoreceptors, shown to be crucial for synaptic transmission to second-order retinal neurons. Localization of other synaptic proteins investigated as well as morphology were unaffected in the developing but fully functional *cacna2d4b* mutant zebrafish retina, suggesting that impaired synaptic function might precede symptoms of cone dystrophy caused by *CACNA2D4* mutation in human patients.

6.2 Introduction

At the first retinal synapse, photoreceptors transmit visual information to bipolar and horizontal cells, the second order neurons of the vertebrate retina. To transmit graded light responses over a broad range of light intensities and long periods of time, photoreceptors, rely on highly elaborate and structurally complex ribbon synapses, harboring large numbers of glutamate-containing vesicles (Matthews and Fuchs, 2010; Mercer and Thoreson, 2011).

At the photoreceptor synaptic terminal, tonic release of vesicles is mediated by Ca^{2+} influx through L-type Ca^{2+} channels. L-type and other high-voltage activated Ca^{2+} channels are complexes of pore-forming α_1 and accessory $\alpha_2\delta$ and β subunits (Simms and Zamponi, 2014). Since α_{1F} (or Cacna1f) is the principal α_1 subunit required for vesicle release from photoreceptors terminals, and it has additionally been proven important for development and maintenance of photoreceptor ribbon synapse organization (Morgans, 2001; Nachman-Clewner, St Jules, and Townes-Anderson, 1999; Bech-Hansen et al., 1998; Chang et al., 2006; Strom et al., 1998; Zabouri and Haverkamp, 2013; Liu et al., 2013; Jia et al., 2014). While α_1 subunits set the principal electrophysiological properties of Ca^{2+} channels, the accessory subunits can modulate kinetics and voltage dependence and play important roles in trafficking of Ca^{2+} channels to the synaptic membrane (Dolphin, 2012; Dolphin, 2013). Of the four distinct $\alpha_2\delta$ and β subunits present in mammals, β_2 (or Cacnb2) and $\alpha_2\delta_4$ (or Cacna2d4) associate with Cacna1f in photoreceptors (Lee et al., 2015; Ball et al., 2002) and function in photoreceptor synaptic transmission (Ball et al., 2002; Ruether et al., 2000).

Important functions of the accessory $\alpha_2\delta_4$ subunit have been demonstrated in the retina of both mice and humans. Human patients carrying homozygous mutations in the *CACNA2D4* gene, encoding $\alpha_2\delta_4$, have been diagnosed with retinal dysfunctions (Ba-Abbad et al., 2016; Wycisk et al., 2006b; Vincent et al., 2014; Huang et al., 2015). Patients show distinctive cone dysfunction, while rod function is either normal or only slightly reduced. In the initial study identifying mutated *CACNA2D4* as a cause for retinal dysfunction in humans (Wycisk et al., 2006b; Ba-Abbad et al., 2016; Vincent et al., 2014; Huang et al., 2015), 2 patients have been diagnosed with slowly progressing cone dystrophy, but a recent study suggests likely non-progressive cone dysfunction in two other patients(Ba-Abbad et al., 2016).

Like human patients, mice carrying a nonsense mutation in *cacna2d4* show distinctively altered photoreceptor responses (Ruether et al., 2000; Wycisk et al., 2006a). However, the phenotype in mice appears more severe than in human patients, since cone photoreceptor responses are completely absent, and rod responses are markedly reduced in electroretinographic measurements. In addition, retinal layers show marked thinning (Ruether et al., 2000; Wycisk et al., 2006a), whereas in human patients, retinal layering appeared normal (Ba-Abbad et al. 2016; Vincent et al. 2014). Studies on the mouse model have shown that cacna2d4 mutation causes disorganization of photoreceptor synapses, demonstrated by abnormal structure of their ribbons (Wycisk et al., 2006a) and mislocalization of synaptic proteins (Caputo et al., 2015). It has also been suggested that VGCC α_1 subunits interact with a Ca^{2+} dependent Cl^- channel (TMEM-16) in the retina, both of which mislocalize as a consequence of absent Cacna2d4 protein, resulting

in impaired Ca^{2+} activated Cl^- currents in rod photoreceptors (Caputo et al., 2015).

Although we are thus starting to understand the mechanisms leading to photoreceptor dysfunction caused by *CACNA2D4* mutation, it is not known which other proteins of the photoreceptor-bipolar cell synapse depend on presence of CACNA2D4. Furthermore, it is still unclear how the symptoms develop, and whether retinal dysfunction is stationary or progressive. Another interesting question is, whether cones and rods are both initially affected by loss of CACNA2D4 or whether dysfunction of one photoreceptor type is a consequence of degeneration of the other.

The zebrafish is a promising model organism to help answer these questions. Like humans, zebrafish heavily rely on day-light vision. Their cone-dominant retina is particularly suited to study cone vision, which seems to be primarily affected in human patients carrying *CACNA2D4* mutations. Furthermore, accessibility during development and its rapidly developing visual system make the zebrafish an attractive model to study retinal function and dysfunction during course of development.

In this study, we identify two zebrafish homologues to mammalian *cacna2d4* genes, termed *cacna2d4a* and *cacna2d4b*. We show that both paralogues have partially overlapping expression patterns in both the developing and the adult zebrafish retina. Using highly efficient CRISPR/Cas9 genome editing, we have generated *cacna2d4a* and *cacna2d4b* mutant zebrafish lines. Phenotypic analysis in the developing but fully functional visual system reveals normal retinal morphology in both mutant lines. Interestingly, the Ca^{2+} channel α_1 subunit important for photoreceptor function mislocalizes in homozygous *cacna2d4b* mutants, while other proteins important for synaptic transmission between photoreceptors and bipolar cells do not display marked abnormalities in abundance or localization. Our findings suggest that incorrect synaptic organization at the molecular level might precede the symptoms of retinal degeneration found in mice and human patients carrying *CACNA2D4* mutations. Ongoing work focuses on phenotypic analysis of further developmental stages and the adult retina, and assessment of retinal function using Electroretinography.

6.3 Methods

6.3.1 Fish husbandry

Zebrafish were kept at 26°C under a 14/10-hour light/dark cycle as previously described (Mullins et al., 1994). Embryos were raised at 28°C in E3 medium and staged according to development in days post fertilization (Kimmel et al., 1995). All fish used for this study were from the Tü wildtype strain.

6.3.2 Bioinformatic analysis of Cacna2d family members

Human and mouse sequences were used as query for Blast searches against the zebrafish genome assemblies zv9 and zv10 on the Ensembl genome browser (`www.ensembl.org`). Predicted annotated proteins with similarity to mammalian *cacna2d* genes that could be retrieved from zv10 assembly were ENSDARP00000096745, ENSDARP00000109719, ENSDARP00000132

Amplicon	Primer name	Primer sequence
cacna2d4a, 1439bp	cacna2d4a_ dr_ -0039s	GGACCCATTCTGATATCAAGC
	cacna2d4a_ dr_ 1400as	ATCAACAGCAAACTCTCGG
cacna2d4b, 602bp	cacna2d4b_ dr_ 0870s	CACTGATTATGTGAGCTACG
	cacna2d4b_ dr_ 1472as	GTTCCCACAACACCTAGC
pT7-gRNA insert	pT7-gRNA_ fw	CAGCTATGACCATGATTACG
	pT7-gRNA_ rev	AAAAGCACCGACTCGGTG
Cacna2d4a	cacna2d4a_ CRISP1_ fw	GGAGAAAGGTACAGATATTTAATCG
CRISPR	cacna2d4a_ CRISP1_ rev	CCAGCATGTTCTCCATTTCC
target region	cacna2d4a_ dCAP_ fw	ATAGAAATATAAAGAAGTGGAACCCATCGTCAAGC
	cacna2d4a_ dCAP_ rev	AAAGCTTTCAACAAATAAACGAAATG
Cacna2d4b	cacna2d4b_ CRISP2_ fw	TAGTTTAGTCCCTGATTTATCCG
CRISPR	cacna2d4b_ CRISP2_ fw2	AAATTCAAGCAAATGCATATAAAATAC
target region	cacna2d4b_ CRISP2_ rev	GCGATAACATTGACAAAGTCG

TABLE 6.1: **List of all primers used in this study**

438, ENSDARP00000113230, ENSDARP00000001281, ENSDART00000136 069, ENSDART00000048023, and ENSDARP00000006267. Two other proteins, ENSDARP00000066454 and ENSDARP00000080995, were only predicted to be translated by the zv9 but not the more recent zv10 assembly. It is therefore possible that they have been wrongly annotated in zv9 due to the presence of pseudogenes. We nevertheless included these two proteins in our phylogenetic analysis.

Phylogenetic analysis was performed on the Phylogeny.fr platform (Dereeper et al., 2008; Dereeper et al., 2010). Briefly, sequences were aligned using the MUSCLE algorithm, ambiguous regions were removed using Gblocks and conserved amino acids were subjected to PhyML maximum likelihood phylogeny. The resulting phylogenetic tree was rendered using TreeDyn.

Presence of known protein domains was analyzed using SMART domain prediction on `http://smart.embl-heidelberg.de`. Proteins were analyzed for presence and position of signal peptides using PrediSi on `http://www.predisi.de/home.html`, and for presence of omega sites and likelihood of GPI anchoring using PredGPI on `http://gpcr.biocomp.unibo.it/predgpi` (Pierleoni, Martelli, and Casadio, 2008).

6.3.3 RNA *in-situ* hybridization on whole mount larvae and adult retinal sections

Fragments of *cacna2d4a* and *cacna2d4b* coding sequence were amplified from cDNA of 5dpf zebrafish by PCR (Jumpstart Taq polymerase, Sigma-Aldrich). A *cacna2d4a* fragment was amplified with primers cacna2d4a_ dr_ -0039s and cacna2d4a_ dr_ 1499as, and a *cacna2d4b* fragment with primers cacna2d4b_ dr_ 0870s and cacna2d4b_ dr_ 1472as (Table 6.1). PCR products were cloned into the pCRII-TOPO vector (Invitrogen). Identity of the cloned fragments was confirmed and orientation was checked by restriction digestion and sequencing. Plasmids were linearized using appropriate restriction enzymes, and in vitro transcription of antisense and sense RNA probes was performed using the Roche DIG-RNA Labeling Kit (Roche).

For whole mount *in-situ* hybridization, embryos were treated with 3 µM PTU (1-phenyl-2-thiourea, Sigma-Aldrich) to suppress pigmentation. Larvae were fixed in 4% paraformaldehyde overnight at 4°C. Whole mount

gene	Target site	Oligo fw	Oligo fw
cacna2d4a	GGAACCCATCGTCAAGATGG	TAGGAACCCATCGTCAAGATGG	AAACCCATCTTGACGATGGGTT
cacna2d4b	GGATGTGAGCGGCAGCATGA	TAGGATGTGAGCGGCAGCATGA	AAACTCATGCTGCCGCTCACAT

TABLE 6.2: **CRISPR target sites and oligonucleotides used for generating sgRNA templates**

in-situ hybridization was carried out as described (Thisse and Thisse, 2008) with modifications (Haug et al., 2013).

For *in-situ* hybridization on adult retinal sections, zebrafish were euthanized using tricaine (MS-222, Sigma-Aldrich). Eyes were excised and fixed in 4% paraformaldehyde overnight at 4°C. Tissue was washed twice in PBS for 5 minutes at room temperature, incubated in 30% sucrose overnight at 4°C, embedded in in Richard-Allan Scientific Neg-50 Frozen Section Medium (Thermo Fisher Scientific) and cryosectioned at 16 µm thickness. *In-situ* hybridization on sections was performed as described (Haug et al., 2013). Sections were coverslipped with Kaiser's glycerol gelatin (Merck). Whole mount and section *in-situ* hybridization experiments were carried out using antisense probes. Control experiments using sense probes resulted in no signal.

6.3.4 Generation of sgRNAs

CRISPR target sites of the GGN18 motif favourable for in-vitro transcription with T7 polymerase (Gagnon et al., 2014) were detected using the ZIFIT CRISPR/Cas oligonucleotide designer on `www.zifit.partners.org`. Potential target sites were rated based on GC content (minimum 50%, (Gagnon et al., 2014) , potential OFF target sites which were manually analyzed by aligning suggested target sites to the zebrafish genome with attention to specificity rules suggesting intolerance to mismatches in the last 12 bases (Le Cong et al., 2013) by BLAT to genome assembly zv9 on the ensembl genome browser (`www.ensembl.org`) and position in open reading frames (the closer to the translational start site the better, but not in the first exon). Oligonucleotides for cloning sgRNA templates (Table 6.2) were retrieved from the ZIFIT tool and ordered at Microsynth. Forward and reverse oligonucleotides were annealed and cloned into pT7-gRNA vector as described (Jao, Wente, and Chen, 2013). Correct insertion of sgRNA templates was confirmed by sequencing. sgRNA template was amplified using a proofreading polymerase (Phusion High Fidelity DNA Polymerase, New England Biolabs) and primers pT7-gRNA_ fw and pT7-gRNA_ rev (Table 6.1). sgRNAs were synthesized from 200-300 ng purified PCR products using the Megascript T7 kit (Ambion) and purified using the MAGAclear kit (Ambion).

6.3.5 Microinjection of Cas9/sgRNA complexes

Cas9-GFP protein (Jinek et al., 2013) was kindly provided by Prof. Dr. Mosimann and Prof. Dr. Martin Jínek. Injection mixes containing 150 ng/µl sgRNA, 832 ng/ µl Cas9-GFP protein and 300 mM KCl were incubated for 10 min at 37°C to allow formation of sgRNA/Cas9-GFP complexes. 1-cell-stage zebrafish embryos were injected with 1 nl of the injection mix.

6.3.6 Mutation analysis

For genotyping injected zebrafish, around 10 embryos from each injected clutch were pooled at 1 to 2 dpf. Only embryos developing normally were used for analysis. For analyzing mutations transmitted through the germline, fin biopsies were obtained from individual adult F1 fish. Tissue was lysed in 25mM NaOH / 0.2mM EDTA for 30 min at 95°C, followed by addition of an equal volume of 40 mM Tris-HCl (pH 5) for neutralization. The lysate was used as template for amplifying targeted regions of *cacna2d4a* (primers cacna2d4a_ CRISP1_ fw cacna2d4a_ CRISP1_ rev) and *cacna2d4b* (primers cacna2d4b_ CRISP2_ fw and cacna2d4b_ CRISP2_ rev) by PCR (Table 6.1), followed by cloning into pCRII-TOPO (Invitrogen). Plasmids isolated from individual clones were analyzed by sequencing of their inserts. For each injected clutch, 8-21 clones, and for each individual F1 fish, 3-6 clones were sequenced. Mutations in injected fish were visualized by a software developed specifically for analyzing CRISPR/Cas9 induced mutations by Dr. Helen Lindsey (Robinson group at IMLS, University of Zurich) (Figures 6.S2, 6.S3), and mutations in the F1 generation were analyzed using the CLC main workbench 7 (Figures 6.S4, 6.S5)

6.3.7 Genotyping of mutant lines

For genotyping offspring of fish with known mutations (F2 and subsequent generations), we employed restriction enzymes cutting only DNA amplified from wild type, but not mutant alleles.

For genotyping fish of the *cacna2d4a* mutant line, the mutated region was amplified using primers Cacna2d4a_ dCAP_ fw and Cacna2d4a_ dCAP_ rev (Table 6.1). dCAPs primers were designed using the dCAPs finder 2.0 on `http://helix.wustl.edu/dcaps/dcaps.html` (Neff, Turk, and Kalishman, 2002). The Cacna2d4a_ dCAP_ fw primer introduces a mismatch generating an AluI restriction site in the wild type allele only (Figure 6.S6). For genotyping fish of the *cacna2d4b* mutant line, where mutation results in removal of an NlaIII restriction site (Figure 6.S7), the mutated region was amplified using Cacna2d4b_ CRISP2_ fw2 and Cacna2d4b_ CRISP2_ rev primers (Table 6.1). PCR products were cut with AluI or NlaIII to genotype fish of the *cacna2d4a* and *cacna2d4b* mutant lines, respectively. Restricted PCR products were analyzed by electrophoresis on a 3% agarose gel.

6.3.8 Histology

5 dpf larvae were were fixed overnight in 4% paraformaldehyde at 4°C and embedded in Technovit 7100 (Kulzer Histotechnik), and cross sections of 3 µm thickness were cut on a microtome (LeicaRM2145, Leica Microsystems). Sections were stained in Richardson solution (1% methylene blue, 1% borax) for 20 seconds, washed for 10 min in ddH_2O and coverslipped with Entellan mounting medium (Merck).

6.3.9 Immunohistochemistry

5 dpf larvae were fixed for 2 hours in 4% paraformaldehyde at 4°C. Section immunohistochemistry was carried out as described previously (Rinner et al., 2005), except that tissue was embedded in Richard-Allan Scientific Neg-50 Frozen Section Medium (Thermo Fisher Scientific) and cryosectioned at 16 µm thickness. Primary antibodies were diluted as follows: rabbit anti-Cacna1fa (1:6000, kindly provided by Michael Taylor, School of Pharmacy, University of Wisconsin-Madison), rabbit anti-RibeyeA (1:2500), rabbit anti-RibeyeB (1:250, both Ribeye antibodies were kindly provided by Teresa Nicholson, Oregon Health & Science University), rabbit anti-mGluR6b (1:750; (Huang et al., 2012)), rabbit anti-EAAT7 EP35 (1:400; (Maurer, 2010) and mouse anti-Zpr-1 (1:400; Zebrafish International Resource Center).

Goat anti-rabbit and anti-mouse IgG conjugated to Alexa 488, 568 or 647 fluorophores (Molecular Probes, Life Technologies), were used at dilutions 1:500 - 1:1000. BODIPY TR Methyl Ester (Molecular Probes, Life Technologies; 1:300) was applied for 20 min after secondary antibodies. Sections were coverslipped with Mowiol (Polysciences) mounting medium containing DABCO (Sigma-Aldrich).

6.3.10 Image acquisition and processing

Images of *in-situ* hybridization experiments and histology experiments were acquired on a wide field light microscope (Olympus BX61) equipped with a Color Camera (ColorViewIII, Soft Imaging System, Olympus). *In-situ* hybridization experiments were imaged using DIC optics and histology experiments in the bright field mode. Images of immunohistochemistry experiments were acquired on a Leica HCS LSI confocal microscope equipped with a 40x oil objective (Leica Microsystems). Images were adjusted for brightness and contrast using ImageJ. Figures were composed with Adobe Photoshop.

6.4 Results

6.4.1 Identification and analysis of two zebrafish *CACNA2D4* homologues

We used verified human and mouse sequences of $\alpha_2\delta$ Ca^{2+} channel subunits to search the zebrafish genome database for $\alpha_2\delta$ subunit family members. While mouse and human genomes contain four $\alpha_2\delta$ subunits (Cacna2d1, Cacna2d2, Cacna2d3 and Cacna2d4), we found a total of 8 genes encoding $\alpha_2\delta$ subunits in the zebrafish genome. However, two of them, which were finally assigned as Cacna2d1a and Cacna2d3b, were not annotated in the most recent genome assembly (zv10 on enseml.org) and might therefore be pseudogenes. Phylogenetic analysis (Figure 6.1A) clearly revealed two zebrafish orthologues to mammalian Cacna2d4, termed Cacna2d4a and Cacna2d4b. Genes encoding these two proteins are located on different chromosomes (*cacna2d4a* on chromosome 4 and *cacna2d4b* on chromosome 25) and are therefore likely products of teleost-specific whole genome duplication, which occurred around 320 -

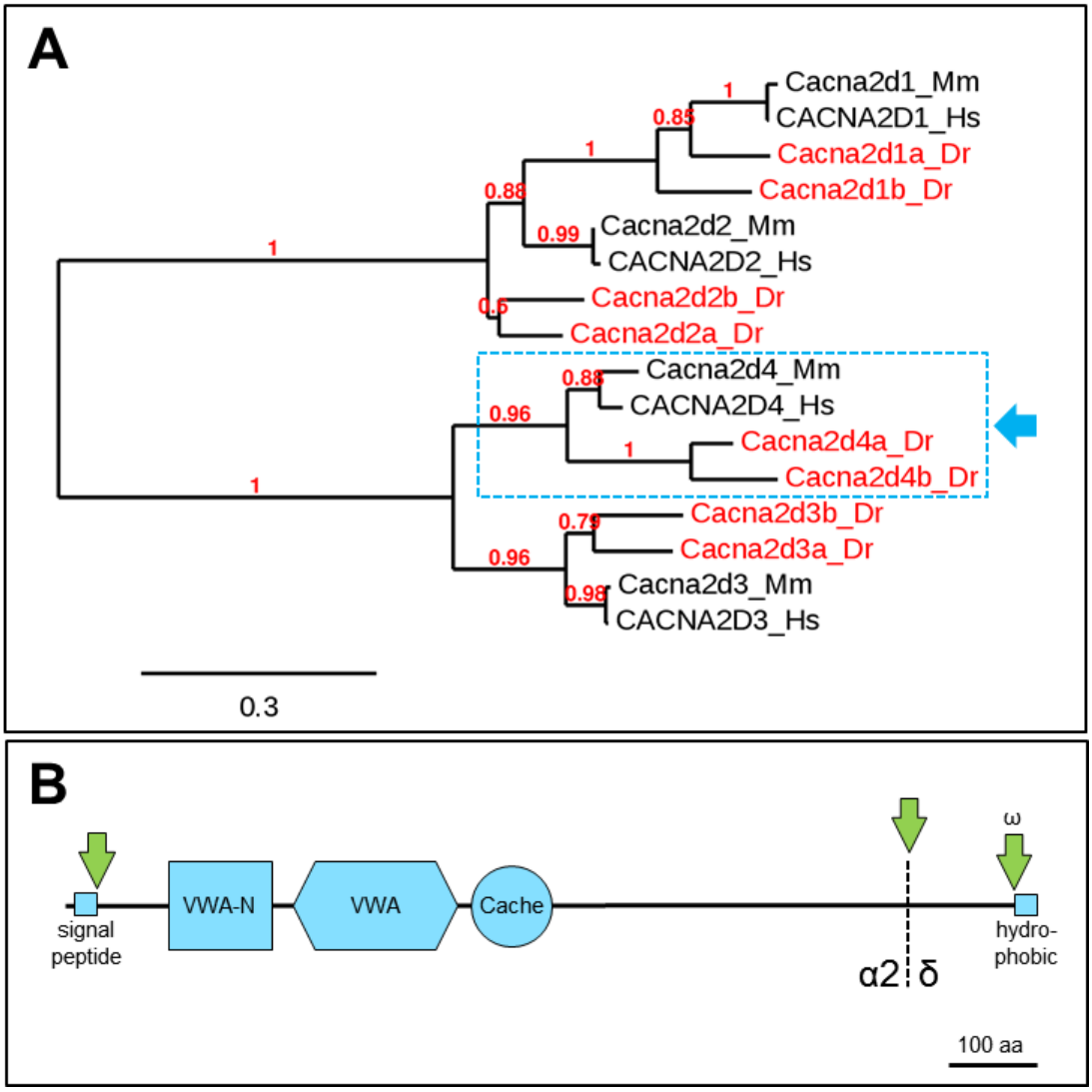


FIGURE 6.1: **Bioinformatic analysis of Cacna2d proteins.** **(A)** Maximum likelihood phylogeny of the Cacna2d family. 8 Cacna2d family members were identified in the zebrafish genome, in contrast to the 4 Cacna2d4 members known from mammals. Mammalian Cacna2d4 clearly has two orthologues in zebrafish (dashed box). **(B)** Schematic representation of predicted protein structure applying to all members of the Cacna2d4 subclade (dashed box in **(A)**). Cacna2d4 proteins contain Van-Willebrand factor (VWA) and VWA N-terminal (VWA-N) domains, as well as one Cache domain. Also an N-terminal signal peptide required for membrane targeting and proteolytic cleavage was identified, as well as ω site adjactent to a short C-terminal hydrophopic region, very likely mediating proteolytic cleavage and GPI anchoring. Proteolytic cleavage of the Cacna2d4 pro-form (dashed line) additionally gives rise to the α_2 and δ peptides, which remain attached to each other through disulfide bonds in mature $\alpha_2\delta$ proteins. Arrows in **(B)** indicate proteolytic cleavage. aa, amino acids; Dr, *Danio rerio*; Hs, *Homo sapiens*; Mm, *Mus musculus*.

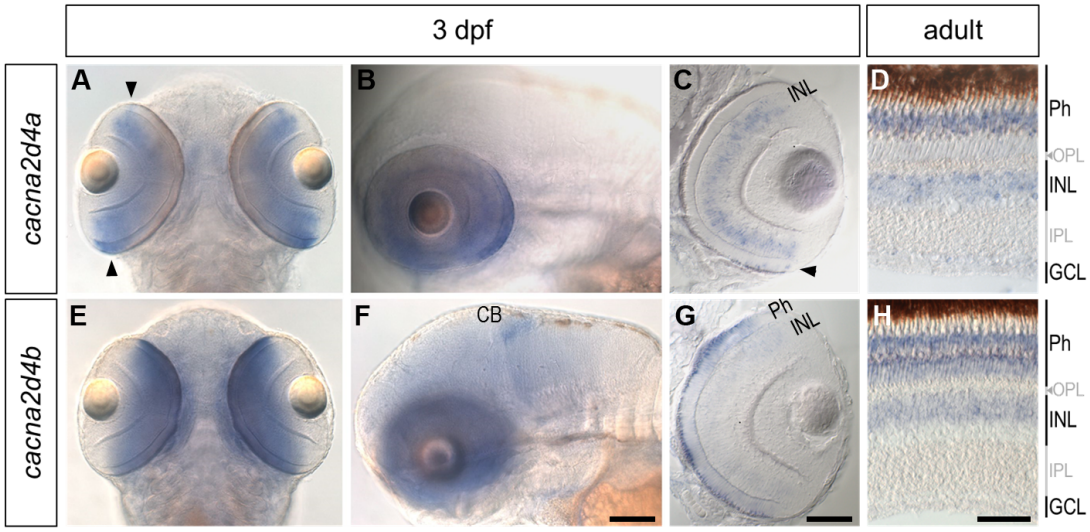


FIGURE 6.2: Expression analysis of zebrafish *cacna2d4a* and *cacna2d4b*. **(A-C)** In 3 days post fertilization (dpf) zebrafish, *cacna2d4a* is expressed throughout the retinal inner nuclear layer (INL) and in photoreceptors of the retinal periphery (arrowheads). **(D)** In the adult retina, *cacna2d4a* is expressed in the INL and the distal half of the photoreceptor layer. **(E-G)** In 3 dpf zebrafish, *cacna2d4b* shows strong expression in photoreceptors **(E,G)**, weak expression in the INL **(G)** and additional signal in the cerebellum **(F)**. **(H)** In the adult retina, *cacna2d4b* is expressed across the photoreceptor layer and in the INL. CB, cerebellum; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; Ph, photoreceptor layer. Scale bars correspond to 100 µm (**F**, applies to **A,B,E,F**) and 50 µm (**G**, applies to **C,G**; **H**, applies to **D,H**).

350 million years ago (Glasauer and Neuhauss, 2014). Cacna2d4a and Cacna2d4b display 60% and 57% amino acid sequence identity to human CACNA2D4, respectively, and 71% identity to one another (for alignment, see Supplementary Figure 6.S1).

$\alpha_2\delta$ subunits are extracellular, GPI anchored proteins (Davies et al., 2010) associating with α_1 subunits through still unknown interaction sites. All $\alpha_2\delta$ subunits analyzed to date contain a Von Willebrand Factor A (VWA) domain, which is known to be involved in binding to cell adhesion and extracellular matrix proteins. Also two Cache domains have been identified in $\alpha_2\delta$ subunits, displaying homology to bacterial chemotaxis proteins (Anantharaman and Aravind, 2000). To gain more insight into possible functional conservation between mammalian and zebrafish Cacna2d4 proteins, we performed prediction of protein domains. All four proteins analyzed (human CACNA2D4, mouse Cacna2d4, zebrafish Cacna2d4a and Cacna2d4b) are predicted to contain a VWA and a VWA N-terminal (VWA-N) domain, as well as one Cache domain (Figure 6.2B). In contrast to one study (Wycisk et al., 2006b), but in agreement with another (Van Den Bossche, Maarten J et al., 2012), a second Cache domain was neither identified

in mammalian nor in zebrafish Cacna2d4 proteins. In addition, all four proteins analyzed were predicted to contain omega sites (Figure 6.2B) required for GPI-anchoring with high confidence.

Therefore, zebrafish Cacna2d4a and Cacna2d4b are, like their mammalian orthologues, probably GPI-anchored extracellular proteins containing domains for interaction with other extracellular proteins.

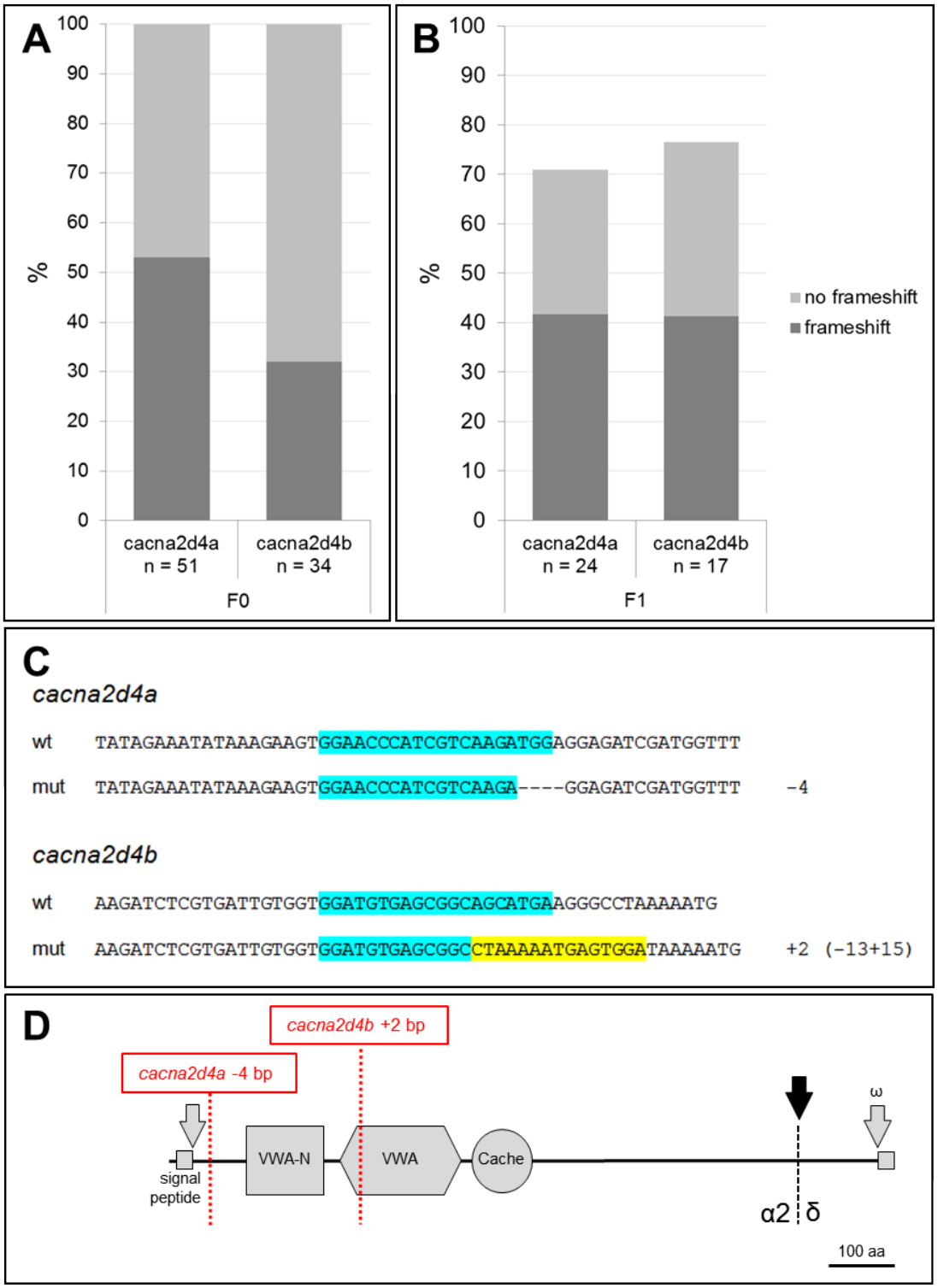
6.4.2 Confined retinal expression of *cacna2d4a* and *cacna2d4b*

In order to analyze expression patterns of *cacna2d4a* and *cacna2d4b* genes, RNA *in-situ* hybridization was performed on larvae and on adult retinal tissue. *cacna2d4a* can be clearly detected in the central inner nuclear layer (INL) of the 3 dpf larval retina, indicative of expression in bipolar cells (Figure 6.2A-C). Additional *cacna2d4a* expression can be detected in small patches of the most peripheral photoreceptor layer (Figure 6.2A,C). *cacna2d4b* shows clear expression throughout the photoreceptor layer (Figure 6.2E,G), while the INL is only weakly stained (Figure 6.2G). In addition to the retina, *cacna2d4b* is also expressed in the cerebellum (Figure 6.2F). Apart from this cerebellar expression, no staining could be detected outside the retina, indicating specialized and confined functions of *cacna2d4a* and *cacna2d4b* in the retina and cerebellum. Expression in 5 dpf larvae is essentially the same as at 3 dpf (not shown). In the adult zebrafish retina, both *cacna2d4a* and *cacna2d4b* are expressed in the central and distal INL (Figure 6.2D,H), and both paralogs are additionally expressed in the photoreceptor layers. However, unlike *cacna2d4b*, which shows staining across the entire photoreceptor layer (Figure 6.2H), *cacna2d4a* is only detected in the distal half (Figure 6.2D), where cone photoreceptors reside. These patterns indicate expression and likely function of *cacna2d4a* only in cones, while *cacna2d4b* is suggested to have functions in both cone and rod photoreceptors.

Since also in mice, *cacna2d4* transcripts are found in photoreceptors and the INL (Postel et al., 2013), our findings demonstrate conserved expression of *cacna2d4* genes between retinae of mammals and fish, making the zebrafish a promising model to study onset and development of retinal defects caused by *cacna2d4* mutations.

6.4.3 Highly efficient mutagenesis of *cacna2d4* genes using CRISPR/Cas9

In order to generate stable zebrafish lines carrying loss-of-function mutations in *cacna2d4a* and *cacna2d4b*, we employed the CRISPR/Cas9 technique, aiming to introduce truncating frameshift mutations early on in the open reading frames. Single guide RNAs (sgRNAs) targeting a region within exon 4 of *cacna2d4a* or exon 8 of cacna2d4b (Table 6.2) were injected as a complex with GFP-tagged Cas9 protein (Jinek et al., 2013) into 1-cell-stage embryos, and mutations were analyzed by cloning and sequencing targeted regions. Injection of *cacna2d4a* and *cacna2d4b* CRISPRs consistently led to highly efficient genome editing, with both yielding mutation rates of 100% (Figure 6.3A). The rate of frameshift mutations was 53% and 32% for *cacna2d4a* and *cacna2d4b* CRISPRs, respectively, and thus indicates preference for in-frame over frameshift mutations. For individual mutations detected, see Supplementary Figures 6.S2 and 6.S3.


A
100
90
80
70
60
50
40
30
20
10
0
%
cacna2d4a
n = 51
cacna2d4b
n = 34
F0
B
100
90
80
70
60
50
40
30
20
10
0
%
cacna2d4a
n = 24
cacna2d4b
n = 17
F1
no frameshift
frameshift
C
cacna2d4a
wt TATAGAAATATAAAGAAGTGGAACCCATCGTCAAGATGGAGGAGATCGATGGTTT
mut TATAGAAATATAAAGAAGTGGAACCCATCGTCAAGA----GGAGATCGATGGTTT -4
cacna2d4b
wt AAGATCTCGTGATTGTGGTGGATGTGAGCGGCAGCATGAAGGGCCTAAAAATG
mut AAGATCTCGTGATTGTGGTGGATGTGAGCGGCCTAAAAATGAGTGGATAAAAATG +2 (-13+15)
D
cacna2d4b +2 bp
cacna2d4a -4 bp
signal peptide
VWA-N
VWA
Cache
ω
α2
δ
100 aa

Injected fish were outcrossed to wildtype, and germline transmission of mutations was determined by genotyping of adult fin biopsies. Mutations were detected in 71% and 76% of *cacna2d4a* and *cacna2d4b* CRISPR F1 fish, respectively (Figure 6.3B, Figure 6.S4, Figure 6.S5), suggesting that germline transmission is not as efficient as mutagenesis itself. Frameshift mutations were detected in 59% and 54% of the *cacna2d4a* and *cacna2d4b* CRISPR F1 fish, respectively. Since this value is comparable to the frameshift rate observed in injected fish, heterozygous loss-of-function mutations in neither *cacna2d4a* nor *cacna2d4b* seem to impair fish survival. F1 fish carrying identical frameshift mutations were incrossed to obtain homozygous, homoallelic mutants in the F2 generation. For the *cacna2d4a* mutant line, a 4 bp deletion (Figure 6.3C) was chosen, by prediction resulting in altered amino acid sequence after position 73 and a stop codon after position 78 (Figure 6.3D). For generating the cacna2d4b mutant line, a -13 / +15 indel mutation (Figure 6.3C) was selected, leading to a predicted altered amino acid sequence after position 258 and a stop codon after position 263 (Figure 6.3D). Since the predicted truncated Cacna2d4a and Cacna2d4b proteins are short and lack conserved domains as well as the C-terminal region essential for membrane targeting, they are expected to be non-functional, in case translation is not prevented by nonsense-mediated mRNA decay in the first place.

6.4.4 Normal retinal morphology in *cacna2d4a* and *cacna2d4b* mutant larvae

Retinal morphology was analyzed on histological sections of 5dpf larval eyes. To ensure exact same age of larvae analyzed and avoid detecting age-related differences, individually genotyped offspring of heterozygous incrosses (see Methods, Page 104) were used for this experiment. Our analysis revealed identical retinal morphology of all three genotypes analyzed (+/+, +/-, -/-) for both *cacna2d4a* and *cacna2d4b* mutant lines (Figure 6.4). Thus,

FIGURE 6.3 *(preceding page)*: **Generation of *cacna2d4a* and *cacna2d4b* mutant zebrafish using CRISPR/Cas9. (A)** Mutation and frameshift rate in CRISPR/Cas9 injected zebrafish embryos. All sequenced alleles were mutated, as determined by sequencing and cloning. n numbers correspond to sequenced clones. Frameshift rates were 53% and 32% for *cacna2d4a* and *cacna2d4b* CRISPR injected fish, respectively. **(B)** Germline transmission of frameshift and in-frame mutations. Mutations were analyzed in adult F1 offspring of CRISPR injected fish crossed to wild type (wt). Mutations were identified in 71% and 76%, of *cacna2d4a* and *cacna2d4b* CRISPR F1 fish, respectively. Of the mutant F1 fish identrified, 59% and 54% carried *cacna2d4a* and *cacna2d4b* frameshift mutations, respectively. n numbers correspond to F1 fish analyzed. **(C)** Frameshift mutations selected for generating stable mutant lines. Blue letters indicate CRISPR target regions, yellow letters insertions and dashes deletions. **(D)** Predicted truncation sites (dashed lines) for proteins resulting from mutated *cacna2d4a* (-4bp) and *cacna2d4b* (+2bp) genes.

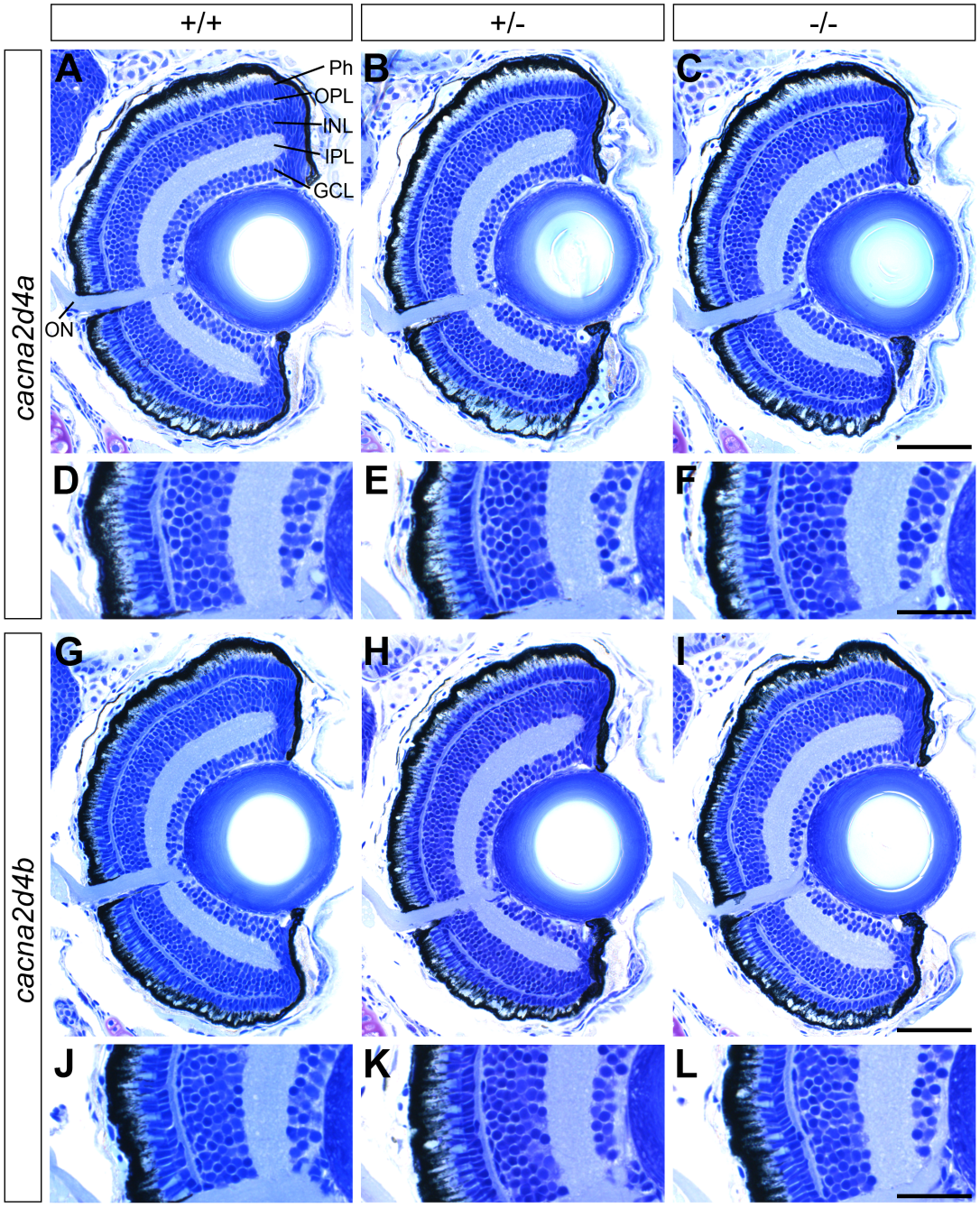


FIGURE 6.4: **Retinal morphology of *cacna2d4a* and *cacna2d4b* mutant 5dpf zebrafish.** Retinal morphology is not affected by hetero- and homozygous *cacna2d4a* **(A-F)** and *cacna2d4b* **(G-L)** mutations. Overall retinal morphology **(A-C, G-I)** as well as thickness of retinal layers **(D-F, J-K)** appear normal. All analyzed larvae are F2 offspring from incrosses of heterozygous F1 parents. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ON, optic nerve; OPL, outer plexiform layer; Ph, photoreceptor layer. Scale bars correspond to 50 µm (**C**, applies to **A-C**; **I**, applies to **G-I**) and 25 µm (**F**, applies to **D-F**; **L**, applies to **J-L**).

it seems that mutation of neither *cacna2d4a* nor *cacna2d4b* does impair retinal development on the gross morphological level. We would like to point out the unchanged thickness of retinal layers even in homozygous mutants, particularly of the outer plexiform layer (OPL), which was reported to be most affected in *cacna2d4* mutant mice (Wycisk et al., 2006a).

6.4.5 *cacna2d4b* mutation causes mislocalization of Cacna1fa, but not of Ribeye proteins

Since $\alpha_2\delta$ subunits of Ca^{2+} channels are known to target α_1 pore-forming subunits to synaptic compartments of the plasma membrane, we tested whether *cacna2d4a* or *cacna2d4b* mutation affects localization of Cacna1fa, a Ca^{2+} channel $\alpha 1$ subunit crucial for cone photoreceptor function in zebrafish (Jia et al., 2014). Since synaptic transmission from photoreceptors to second-order neurons is first fully functional at 5 dpf (Biehlmaier, Neuhauss, and Kohler, 2003), we selected this developmental stage for our analysis. As previously reported (Jia et al., 2014), Cacna1fa exclusively localized to photoreceptor synapses in the 5 dpf retina of wildtype zebrafish (Figure 6.5A,D). While Cacna1fa expression in *cacna2d4a* mutants (Figure 6.5B,E) does not appear different from wt, Cacna1fa is dramatically decreased in *cacna2d4b* mutant fish (Figure 6.5C,F). In addition to decreased protein expression at the photoreceptor synapse, we observed ectopic punctate Cacna1fa staining in mutant *cacna2d4b* photoreceptors, which was essentially never seen in the wildtype retina (Figure 6.6). These results indicate that in zebrafish photoreceptors, Cacna2d4b plays an important role in targeting Cacna1fa to the photoreceptor synapse, and that failure to do so does not only result in decreased synaptic expression, but also in ectopic clustering of Cacna1fa channel subunits.

Absence of Cacna1fa has been shown to impair the formation of synaptic ribbons in zebrafish photoreceptors and cause decreased levels of Ribeye b protein at the photoreceptor synapse (Jia et al., 2014). Ribeye b is the main structural component of photoreceptor ribbon synapses in zebrafish (Wan, Almers, and Chen, 2005), and Ribeye proteins have been generally proven important for formation of ribbon synapses (Regus-Leidig et al., 2009; Magupalli et al., 2008; Sheets et al., 2011).

Therefore, we tested whether decreased Cacna1fa expression caused by *cacna2d4b* mutation also affects Ribeye expression in photoreceptors, using an antibody specific to Ribeye b (Sheets et al., 2011). In the 5 dpf wildtype retina, Ribeye b predominantly localizes to the photoreceptor synapse, but is at lower levels also present outside of the photoreceptor synaptic compartment (Figure 6.5G). No apparent changes in Ribeye b expression can be observed in *cacna2d4a* and *cacna2d4b* mutant zebrafish (Figure 6.5H,I), suggesting that synaptic localization of Ribeye b in the 5 dpf retina is neither dependent on presence of Cacna2d4a or Cacna2d4b, nor on wildtype levels of Cacna1fa.

Since both *cacna2d4a* and *cacna2d4b* are expressed in the INL and might therefore affect ribbon synapses at bipolar cell axon terminals, we also examined localization of Ribeye a, which shows prominent expression in the zebrafish retinal INL (Wan, Almers, and Chen, 2005). Using an antibody specific to Ribeye a (Sheets et al., 2011), we observed clustered localization in the wt INL (Figure 6.5J), most likely representing localization in bipolar

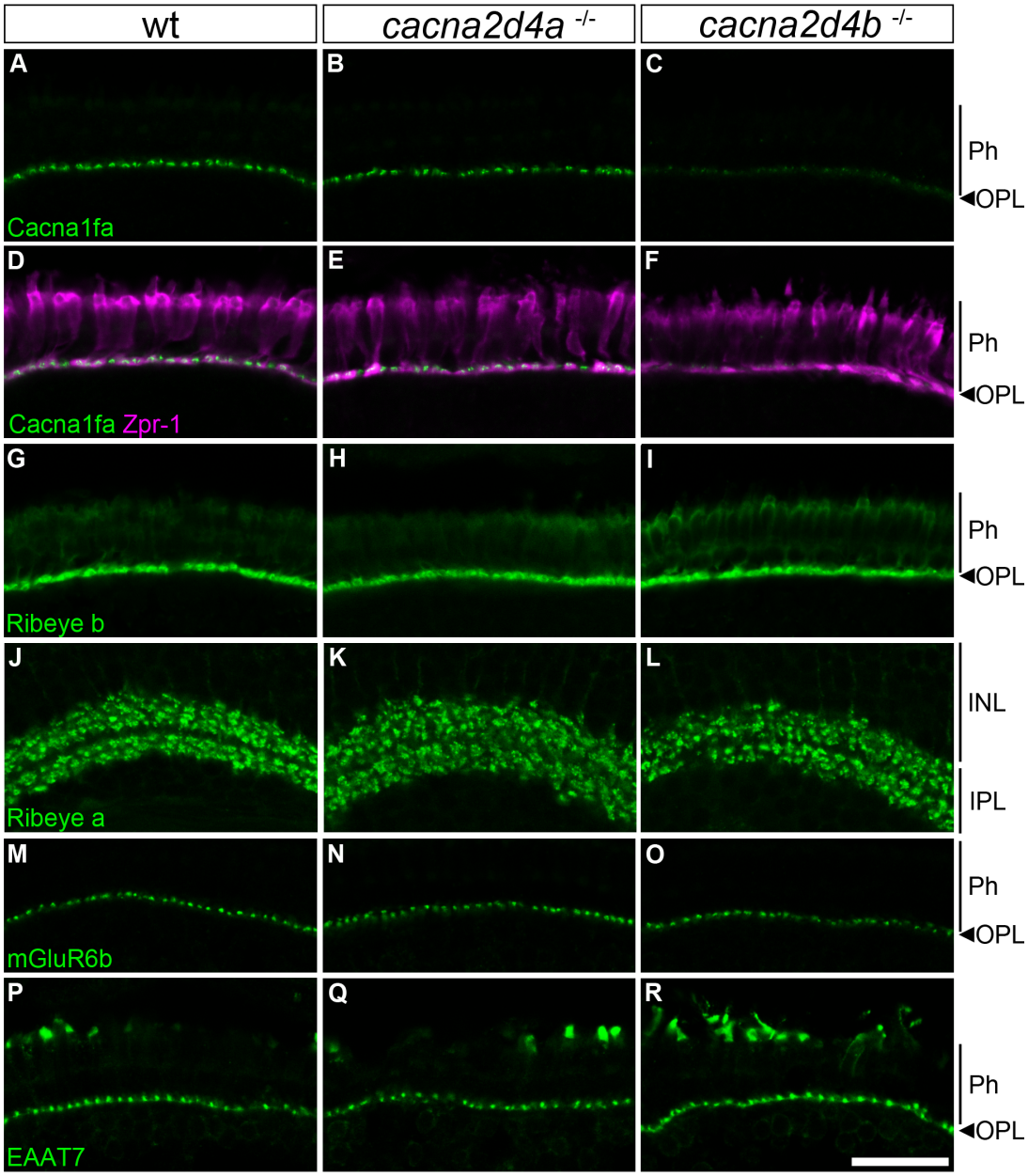


FIGURE 6.5: **Localization of synaptic proteins in the retina of *cacna2d4a* and *cacna2d4b* mutant zebrafish at 5 dpf. (A-F)** Cacna1fa localization at photoreceptor terminals is not affected by *cacna2d4a* mutation **(B,E)**, but dramatically decreased in *cacna2d4b* homozygous mutants **(C,F)**. **(G-I)** Ribeye b localizes to wt photoreceptor terminals at 5 dpf **(G)**, and staining is not altered due to *cacna2d4a* **(H)** or *cacna2d4b* mutation **(I)**. **(J-L)** Also localization of Ribeye a in bipolar cell axon terminals **(J)** is not affected by *cacna2d4a* **(K)** and *cacna2d4b* **(L)** mutations. **(M-R)** mGlur6b and EAAT7 both show punctate staining in the outer plexiform layer (OPL) of *cacna2d4a* **(N,Q)** and *cacna2d4b* **(O,R)** mutant fish, indistinguishable from mGlur6b and EAAT7 wild type localization **(M,P)**. INL, inner nuclear layer; IPL, inner plexiform layer; Ph, photoreceptor layer. Scale bar corresponds to 20 µm.

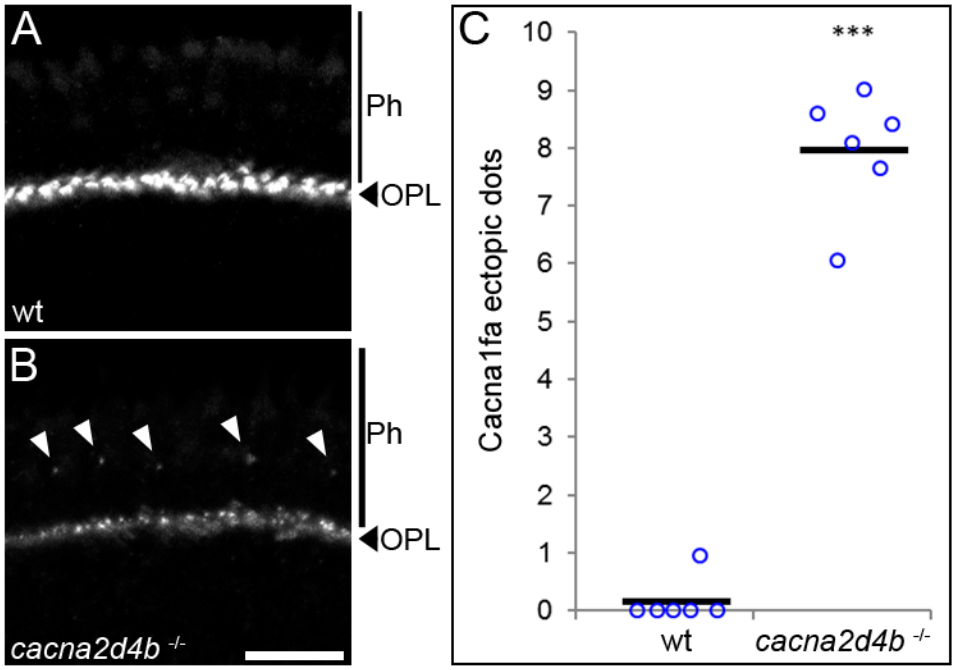


FIGURE 6.6: **Cacna1fa distribution in the 5 dpf *cacna2d4b*$^{-/-}$ retina. (A)** In wt, Cacna1fa staining is exclusively found at photoreceptor terminals in the OPL. **(B)** In the *cacna2d4b*$^{-/-}$ retina, Cacna1fa staining is strongly decreased. Furthermore, photoreceptors show ectopic punctate staining outside the OPL. **(C)** Quantification shows a highly significant difference in number of ectopic Cacna1fa puncta between wt and *cacna2d4b*$^{-/-}$ photoreceptors (two sample t-test assuming unequal variances, p = 2.6E-06). Puncta were automatically counted using 3D Objects Counter in ImageJ on confocal images from 6 wt and 6 cacna2d4b-/- zebrafish. Since lengths of retinal stretches imaged were slightly divergent (89 – 128 µm), numbers of puncta were normalized to 100 µm for each image. Scalebar corresponds to 10 µm.

cell axon terminals. This staining was not altered in *cacna2d4a* and *cacna2d4b* mutant zebrafish (Figure 6.5K,L), suggesting that neither Cacna2d4a nor Cacna2d4b are required for Ribeye a localization to bipolar cell ribbon synapses during development.

6.4.6 Normal localization of postsynaptic proteins involved in bipolar cell ON signaling

We next asked whether postsynaptic components of photoreceptor-bipolar cell synapses are affected by *cacna2d4a* or *cacna2d4b* mutation and therefore investigated the localization of proteins involved in glutamate sensing by bipolar cells. mGluR6 causes the hyperpolarizing response of ON bipolar cells to glutamate binding, and mGluR6 clustering at bipolar cell dendritic tips was shown to be impaired in mice deficient for the Ca^{2+} channel β subunit Cacnb2 (Katiyar et al., 2015). Also excitatory amino acid transporters

(EAATs) participate in bipolar cell ON signaling in fish (Grant and Dowling, 1996; Wong, Adolph, and Dowling, 2005), and such a role has been suggested for the EAAT family member EAAT7 in particular (Maurer, 2010).

Using antibodies specific to zebrafish mGluR6b (Huang et al., 2012) and zebrafish EAAT7 (Maurer, 2010), we observed clear punctate staining in the OPL of 5 dpf wt, *cacna2d4a* mutant and *cacna2d4b* mutant zebrafish (Figure 6.5M-R). Thus, neither Cacna2d4a nor Cacna2d4b appear to be important for proper localization of mGluR6b and EAAT7 in bipolar cell dendritic tips during development.

6.5 Discussion

Mutations in the *CACNA2D4* gene, encoding the $\alpha_2\delta_4$ subunit of voltage-gated Ca^{2+} channels, cause cone dysfunction in human patients (Wycisk et al., 2006b; Vincent et al., 2014; Huang et al., 2015; Ba-Abbad et al., 2016). In order to gain insight into mechanisms leading to onset and progression of retinal defects, we identified homologous zebrafish genes and started to study their functions in the cone-dominant zebrafish retina.

Two orthologues to human *CACNA2D4* exist in the zebrafish genome, termed *cacna2d4a* and *cacna2d4b*. Expression of these two genes is restricted to retina and cerebellum in developing zebrafish. In the developing and adult retina, *cacna2d4a* and *cacna2d4b* show partially overlapping expression patterns in photoreceptors and the INL, similar to *Cacna2d4* expression in the mouse retina. Mutagenesis using the CRISPR/Cas9 system was highly efficient, with mutation rates of 100% for both sgRNAs injected as a complex with Cas9-GFP protein, and frameshift mutations were successfully transmitted through the germ line. Mutation of *cacna2d4b* causes mislocalization of Cacna1fa, the principle pore-forming subunit of voltage-gated Ca^{2+} channel complexes in photoreceptors. Localization of other synaptic proteins was not affected at 5 dpf, and also retinal morphology analyzed at the same age appeared normal.

In summary, our study indicates conserved roles of $\alpha_2\delta_4$ subunits in the retina of mammals and zebrafish, making *cacna2d4* mutant zebrafish a promising disease model for retinal dysfunction caused by *CACNA2D4* mutations.

6.5.1 $\alpha_2\delta_4$ function is restricted to few regions of the CNS

Our expression analysis on 3 dpf zebrafish larvae showed exclusive expression of *cacna2d4a* in the retina, while *cacna2d4b* was additionally expressed in the cerebellum.

Also in the mammalian retina, presence of *Cacna2d4* transcripts, as well as Cacna2d4 protein, has been demonstrated (De Sevilla Muller, Luis Perez et al., 2013; Postel et al., 2013), whereas expression of *Cacna2d4* in the brain is ambiguous (De Sevilla Muller, Luis Perez et al., 2013; Qin et al., 2002; Schlick, Flucher, and Obermair, 2010). The fact that *CACNA2D4* mutations have been identified in patients with late onset bipolar disorder (Van Den Bossche, Maarten J et al., 2012) supports expression in human brain. Probably, mammalian $\alpha_2\delta_4$ is expressed in the brain at low levels or in only few brain areas. The situation appears similar in zebrafish where, apart from

the retina, clear staining could only be observed in the cerebellum for the *cacna2d4b* paralogue.

Expression of *cacna2d4b* in the cerebellum is intriguing, since mutation or removal of mouse *Cacna2d2*, also expressed in the cerebellum, causes epilepsy and cerebellar ataxia (Brill et al., 2004; Ivanov et al., 2004; Brodbeck et al., 2002; Barclay et al., 2001). Although we did not observe an obvious phenotype in our mutant lines, it would be interesting to see whether *cacna2d4b* mutant zebrafish exhibit mild signs of ataxia or epilepsy.

6.5.2 Retinal expression of *cacna2d4* genes is conserved across vertebrates

In the adult zebrafish retina, we found both *cacna2d4a* and *cacna2d4b* to be expressed in the INL and in photoreceptors. In the mouse retina, *Cacna2d4* transcripts were detected in the same retinal layers (Postel et al., 2013), and Cacna2d4 protein was shown to be located in photoreceptors as well as ON bipoar cells, Müller glia and displaced ganglion cells, residing in the INL (De Sevilla Muller, Luis Perez et al., 2013). Thus, it can be concluded that expression patterns of *cacna2d4* genes are basically conserved between fish and mammalian retinae.

Retinal expression patterns of the paralogous *cacna2d4a* and *cacna2d4b* zebrafish genes are similar to each other, but not identical. In the developing zebrafish retina, *cacna2d4a* and *cacna2d4b* are predominantly expressed in cells of the INL and photoreceptors, respectively. Furthermore, expression patterns in the adult photoreceptor layer suggest exclusive expression of *cacna2d4a* in cones, whereas *cacna2d4b* transcripts can be found in both photoreceptor types. Due to these partly overlapping expression patterns, we suggest that at least partial subfunctionalization (Force et al., 1999) has shaped the evolutionary history of *cacna2d4a* and *cacna2d4b* paralogues, which very likely arose as products of teleost-specific whole genome duplication.

6.5.3 Targeting of Ca^{2+} channel complexes by auxiliary $\alpha_2\delta_4$ subunits

Upon mutation of *cacna2d4b*, we found a dramatic decrease of the α_1 subunit Cacna1fa at photoreceptor synaptic terminals. A similar observation was made in mice, where *Cacna2d4* mutation resulted in decrease of α_1 subunits visualized with a pan α_1 antibody (Caputo et al., 2015). Specific mislocalization of the Cacna1f subunit due to *cacna2d4* mutation is however reported here for the first time.

Our findings are in agreement with association of Cacna2d4 and Cacna1f, which has been suggested by several studies due to their colocalization (Mercer and Thoreson, 2011; Lee et al., 2015) and has recently also directly been shown in the mouse retina (Lee et al., 2015).

The role of $\alpha_2\delta$ subunits in trafficking of Ca^{2+} channel complexes is well established, as is their capability to influence biophysical properties of channels (reviewed by Dolphin, 2013). Mammalian Cacna2d4 has specifically been shown to modulate Cacna1f voltage-dependent activation (Lee

et al., 2015). However, due to the strongly impaired localization of α_1 subunits caused by absence of Cacna2d4 shown by this and another study (Caputo et al., 2015), we propose that impairment of retinal function due to *CACNA2D4* mutation is predominantly due to a targeting defect of Ca^{2+} channel complexes important for synaptic transmission between photoreceptors and second-order neurons.

No effect of *cacna2d4a* mutation on Cacna1fa localization could be observed. This is not surprising, since *cacna2d4a* is not expressed in photoreceptors at 5 dpf, when Cacna1fa localization was examined. It is however well possible that Cacna2d4a interacts with Cacna1fa in the adult retina, when both genes encoding these Ca^{2+} channel subunits are expressed in photoreceptors (Jia et al., 2014).

Besides interaction with Cacna1f, it is well possible that Cacna2d4 additionally interact with other α_1 subunits. Cacna1d is another good candidate for mediating vesicle release from photoreceptors, and also bipolar cells. Like Cacna1f, native Cacna1d channels have been shown to lack Ca^{2+} dependent inactivation, although by a different mechanism (reviewed by Simms and Zamponi, 2014). This resistance is likely crucial for mediating tonic glutamate release from photoreceptors and other cells containing ribbon synapses, like bipolar cells and auditory hair cells. Cacna1d is expressed in the mouse retina, with strong signal in the INL and a subset of GCL neurons, and weaker expression in photoreceptors. Knockout results in no obvious retinal phenotype, but slightly altered ERG responses (Wu et al., 2007). In zebrafish, two paralogues to mammalian *Cacna1d* exist, and both of them are expressed in the retina during development (Sidi et al., 2004) and adulthood (the authors, unpublished observation). It remains to be seen whether membrane targeting of Cacna1d is mediated by Cacna2d4 Ca^{2+} channel subunits.

6.5.4 Involvement of $\alpha_2\delta_4$ proteins in development and maintenance of retinal structure and function

In *cacna2d4a* and *cacna2d4b* mutant zebrafish, we observed normal eye morphology and retinal layering at 5 dpf, indicating normal retinal development on the gross morphological level. We also noted that localization of Ribeye b in photoreceptor terminals and Ribeye a in bipolar cell axon terminals is not different from the wildtype retina.

The formation of ribbon synapses has been shown to rely on the presence of Ribeye protein (Regus-Leidig et al., 2009; Magupalli et al., 2008; Sheets et al., 2011), the major structural components of ribbon synapses. The normal appearance of Ribeye localization points to unimpaired ribbon formation in *cacna2d4a* and *cacna2d4b* mutant zebrafish. To make a clear statement about the integrity of ribbon synapses, it will however be necessary to resolve their structure by transmission electron microscopy.

Absence of zebrafish Cacna1fa leads to strong decrease of Ribeye b protein in adult photoreceptor terminals (Jia et al., 2014). It was therefore surprising that severe reduction of Cacna1fa caused by *cacna2d4b* mutation did not result in a similar phenotype. Two possible explanations for this seem likely. First, the low levels of Cacna1fa persisting at the photoreceptor synapse (Figures 6.5C and 6.6B) might be sufficient to ensure correct Ribeye b localization. Second, as we only examined the 5 dpf retina, Ribeye

b localization might be established independently from Cacna1fa during development, but might become perturbed later as a secondary effect of synaptic dysfunction.

Interestingly, in mice lacking functional Cacna1f channels, Ribeye first showed normal abundance in the OPL, but decreased from a certain time point in development (Liu et al., 2013). However, unlike in our study, horseshoe-shaped Ribeye staining never developed in these mice. Another interesting study on Cacna1f deficient mice observed that also scaffolding proteins other than Ribeye are properly targeted at first, but that their expression and localization are not maintained in adulthood (Zabouri and Haverkamp, 2013).

Outgrowth of horizontal and bipolar cell dendrites into the outer nuclear layer is a common feature of photoreceptor synapse defects (Specht et al., 2007). Notably, we observed ectopic Cacna1fa puncta outside of the photoreceptor synaptic compartment in *cacna2d4b* mutant zebrafish, posing the question whether lack of Cacna2d4b leads to ectopic synaptogenesis. Our stainings for postsynaptic proteins of photoreceptor-ON bipolar cell synapses (mGluR6b and EAAT7) indicate that there are no functional ectopic synapses between photoreceptors and ON bipolar cells at 5 dpf. In mice with photoreceptor synaptopathies, ectopic synapse formation in the OPL starts around the age when photoreceptor synapse development is completed (Specht et al., 2007) and progresses over time (Specht et al., 2007; Zabouri and Haverkamp, 2013; Liu et al., 2013). Thus, it will be important to examine ectopic synaptogenesis up to adulthood and also in aging *cacna2d4a* and *cacna2d4b* mutant zebrafish.

6.5.5 Conclusions

In this study, we demonstrate conserved expression and function of auxiliary voltage-gated Ca^{2+} channel $\alpha_2\delta_4$ subunits between mammals and zebrafish. Our newly established *cacna2d4a* and *cacna2d4b* mutant zebrafish lines are therefore promising disease models for cone dysfunction caused by *CACNA2D4* mutation in humans. First results from these$\alpha_2\delta_4$ deficient zebrafish suggest that in human patients, incorrect synaptic organization might precede symptoms of cone dystrophy caused by *CACNA2D4* mutation.

6.6 Outlook

Since our functional data from the *cacna2d4b* mutant line are promising by showing defective synaptic localization of Cacna1fa, a protein crucial for photoreceptor synapse function, it will be exciting to pursue studies on *cacna2d4a* and *cacna2d4b* mutant zebrafish lines.

To correctly interpret experiments on these mutant lines, it will be important to determine absence of protein due to the mutations introduced in *cacna2d4a* and *cacna2d4b*. Although our mutant lines carry truncating frameshift mutations early on in the open reading frames of *cacna2d4a* and *cacna2d4b*, there is a chance that alternative splicing will remove the defective exons, which might lead to translation of partially functional proteins. Consequences of such events are difficult to interpret. Since there are no specific antibodies against zebrafish Cacna2d4a and Cacna2d4b available,

we are going to investigate the effect of *cacna2d4a* and *cacna2d4b* mutations on the mRNA level. Transcripts encoding defective protein are frequently subjected to nonsense-mediated decay, resulting in decrease of mRNA levels, which can be investigated by reverse-transcriptase PCR. We will also specifically check whether transcripts detected contain the mutated exons, since their presence would indicate that alternative splicing, which can potentially lead to translation of partially functional Cacna2d4 protein, is not occurring. In addition, we will attempt immunohistochemistry on *cacna2d4a*/*cacna2d4b* double mutants (see below), using antibodies raised against mammalian Cacna2d4 and expected to recognize both zebrafish Cacna2d4a and Cacna2d4b proteins to see whether staining is abolished.

cacna2d4a and *cacna2d4b* expression patterns are not identical, but they overlap during development and adulthood. Therefore, it is possible that they carry out partially redundant functions in cells co-expressing them. Although further interesting observations are likely going to be made from single knockout of *cacna2d4a* and *cacna2d4b*, double knockouts are preferred to model retinal dysfunction caused by mutation of the non-duplicated human *CACNA2D4* gene. We have started to establish double mutant lines by crossing homozygous *cacna2d4a* and *cacna2d4b* mutants.

All morphological and immunological studies on *cacna2d4a* and *cacna2d4b* mutant zebrafish presented here will be extended to further stages of development and to adulthood. Of particular interest is the retina after signaling from rods has become fully functional, which is the case from 15 dpf onwards, (Branchek and Bremiller, 1984). Also studying the ageing zebrafish retina will be important, since for most human patients carrying *CACNA2D4* mutations, disease onset was reported during adulthood, except from photophobia that was in some cases already apparent during infancy (Ba-Abbad et al., 2016; Huang et al., 2015; Vincent et al., 2014; Wycisk et al., 2006b).

In order to examine potential ectopic synapse formation and sprouting of bipolar cell dendrites into the OPL, we will inject transgenic constructs driving expression of a membrane-tagged fluorescent protein specifically in bipolar cells (see Chapter 4) into *cacna2d4a* and *cacna2d4b* (double) mutant embryos and examine morphology of bipolar cell dendrites over time.

Furthermore, we will assess retinal function using electroretinography (Makhankov, Rinner, and Neuhauss, 2004), and the optokinetic response (reviewed by Huang and Neuhauss, 2008) will be used as read out of visual acuity and other aspects of visual function.

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6.7 Supplementary material

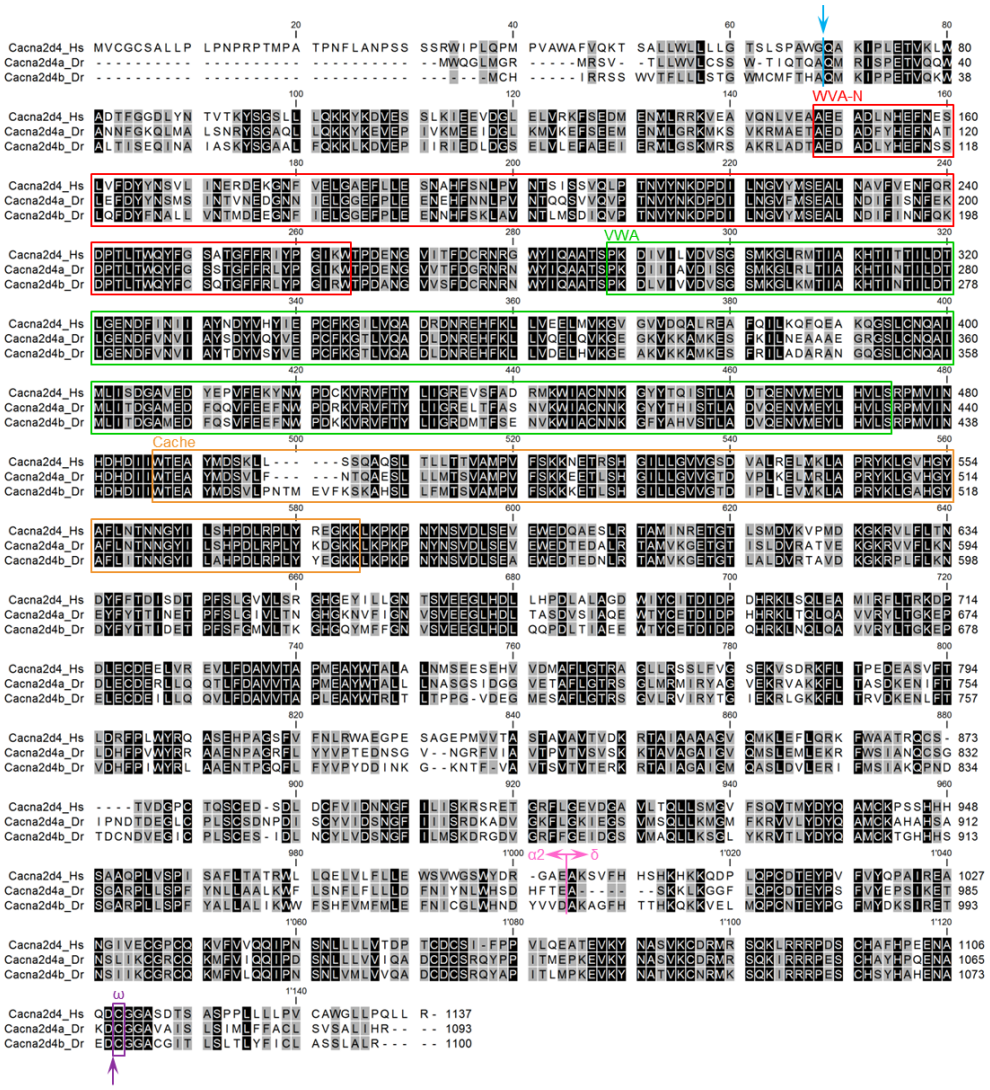

FIGURE 6.S1: **Alignment of Human and Zebrafish Cacna2d4 proteins.** Input sequences were verified human Cacna2d4 and zebrafish proteins deduced from cloned sequences, differing from automated gene annotation in the zv10 genome assembly at 5′ and 3′ ends. Alignment was performed using Clustal Omega (`http://www.ebi.ac.uk/Tools/msa/clustalo`) and visualized on CLC Main Workbench 7. Zebrafish proteins Cacna2d4a and Cacna2d4b display overall 60% and 57% conservation to human Cacna2d4 on the amino acid level, respectively. Amino acid conservation to human protein domains is 73% and 73% in the VWA-N domain, 76% and 74% in the VWA domain and 83% and 78% in the Cache domain for Cacna2d4a and Cacna2d4b, respectively. Arrows indicate sites of proteolytic cleavage at the N-terminus (blue) and at the omega site close to the C-terminus (purple).

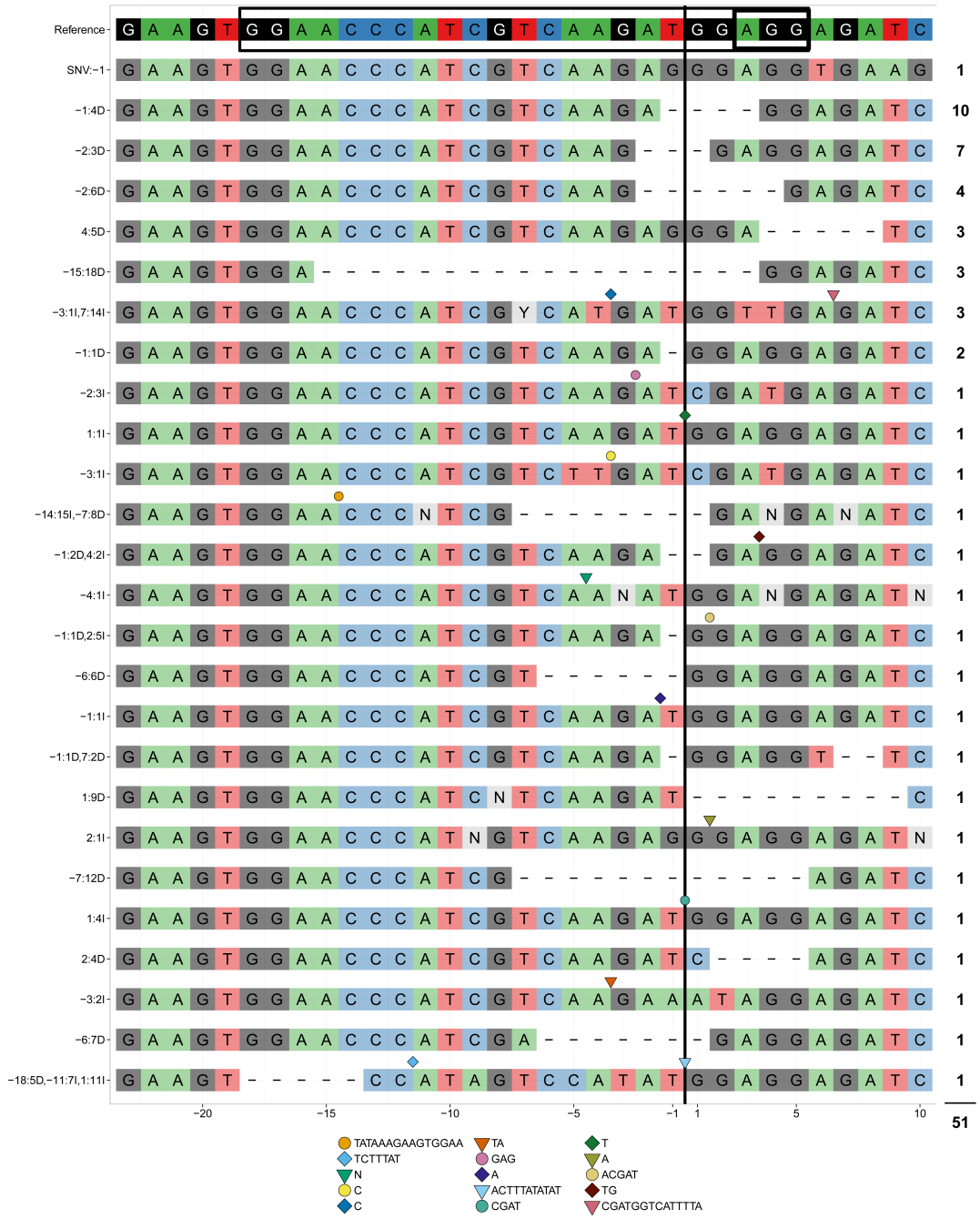

FIGURE 6.S2: **Mutations detected in cacna2d4a CRISPR/Cas9 injected zebrafish embryos.** The target site in the reference wild type allele (top) is indicated by a black box, and the PAM motif adjacent to the target site by a thick black box. A black vertical line represents the predicted Cas9 cutting site. Numbers on the right site indicate how often a certain mutation was found, indicating preferences of certain mutations over others. Dashes represent deletions, and symbols insertions that were not included in the alignment due to space restriction. The corresponding inserted sequences are shown at the bottom.

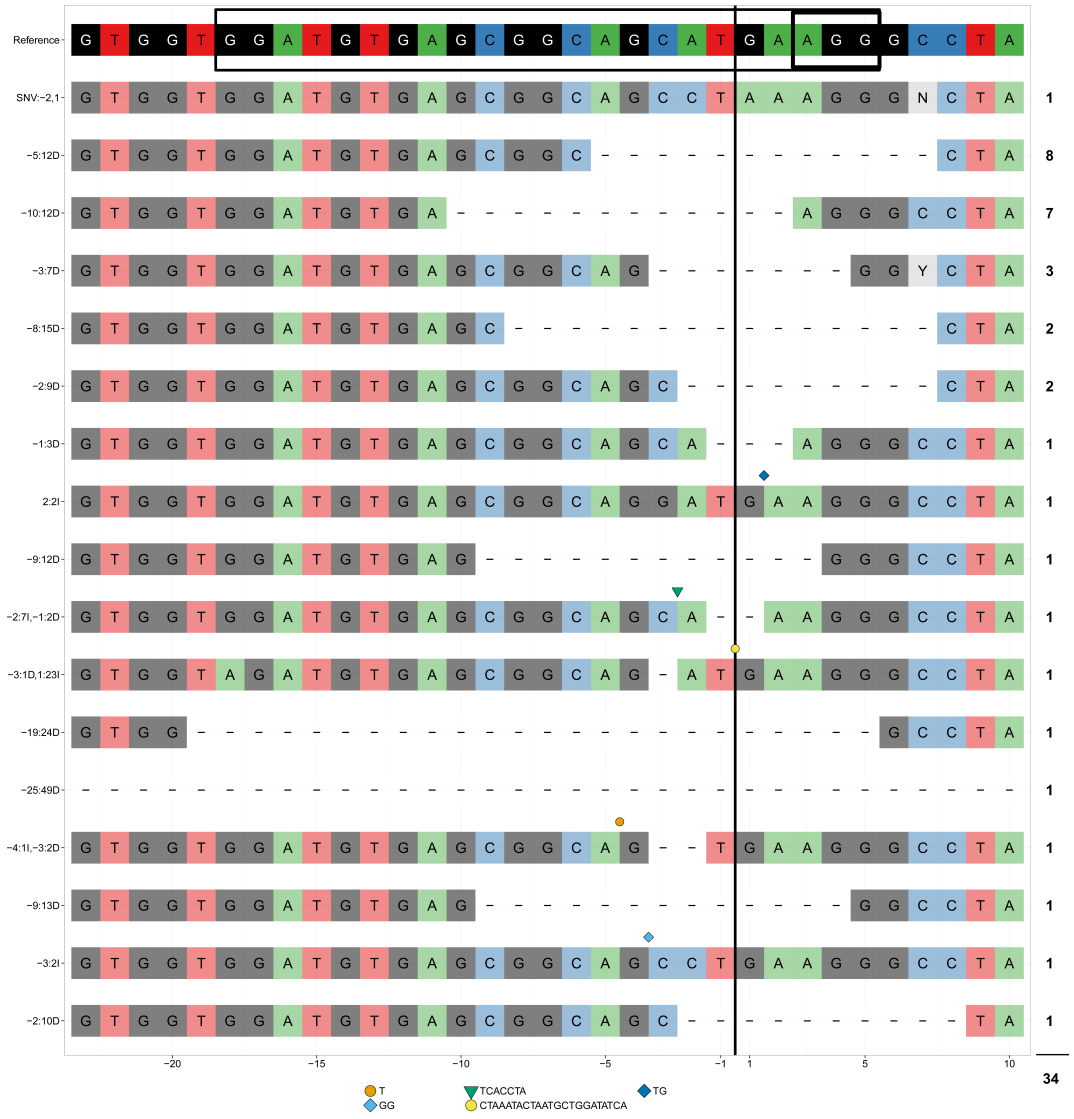

FIGURE 6.S3: **Mutations detected in *cacna2d4b* CRISPR/Cas9 injected zebrafish.** For explanation, see caption of Supplementary Figure 6.S2.

***cacna2d4a* CRISPR F1 genotypting**

wt	TATAGAAATATAAAGAAGTGGAACCCATCGTCAAGATGGAGGAGATCGATGGTTT		
F1#1	TATAGAAATATAAAGAAGTGGAACCCATC------CAGGAGGAGATCGATGGTTT	-6 (-8+2)	(1/6)
F1#2	wt		(0/6)
F1#3	wt		(0/3)
F1#4	wt		(0/5)
F1#5	TATAGAAATATAAAGAAGTGGAACCCATCGTCAAGATCGATGGAGGAGATCGATGGTTT	+4	(1/6)
F1#6	wt		(0/6)
F1#7	wt		(0/5)
F1#8	wt		(0/3)
F1#9	TATAGAAATATAAAGAAGTGGAACCCATCGTCAAGAGATCGAGGAGGAGATCGATGGAGGAGATCGATGGTTT	+18	(3/3)
F1#10	TATAGAAATATAAAGAAGTGGAACCCATCGTCAAGAGATCGAGGAGGAGATCGATGGAGGAGATCGATGGTTT	+18	(1/3)
F1#11	TATAGAAATATAAAGAAGTGGAACCCATCG--------GAGGAGATCGATGGTTT	-8	(3/3)
F1#12	TATAGAAA---------GGTCGGATCCTCCGGATCCTGGAGGAGATCGATGGTTT	-9 (-28+19)	(2/3)
F1#13	wt		(3/3)
F1#14	TATAGAAATATAAAGAAGTGGAACCCATCGTCAAGAGATCGAGGAGGAGATCGATGGAGGAGATCGATGGTTT	+18	(2/3)
F1#15	TATAGAAATATAAAGAAGTGGAACCCATCGTCAAGA----GGAGATCGATGGTTT	-4	(2/3)
F1#16	TATAGAAATATAAAGAAGTGGAACCCATCGTCAAGAGGAGGAGGAGGTCGATGGTTT	+2 (-9+11)	(3/3)
F1#17	TATAGAAATATAAAGAAGTGGAACCCATCGTCAAGATGG----------TTT	-13	(2/3)
F1#18	TATAGAAATATAAAGAAGTGGAACCCATCGTCAAGAGGAGGAGAAAGGAGATCGATGGTTT	+6 (-3+9)	(2/3)
F1#19	TATAGAAATATAAAGAAGTGGAACCCATCGTCAAGAGATCTGGAGGAGATCGATGGTTT	+4	(1/2)
F1#20	TATAGAAATATAAAGAAGTGGAACCCATCGTCA----------GATGGTTT	-14	(6/7)
F1#21	TATAGAAATATAAAGAAGTGGAACCCATCGTCAAGA----GGAGATCGATGGTTT	-4	(3/3)
F1#22	TATAGAAATATNAAGAAGTGGAACCCATCGTCA----------GATGGTTT	-14	(3/3)
F1#23	TATAGAAATATAAAGAAGTGGAACCCATCGTCAAGA----GGAGATCGATGGTTT	-4	(3/3)
F1#24	TATAGAAATATAAAGAAGTGGAACCCATCGTCAAGA----GGAGATCGATGGTTT	-4	(2/3)

FIGURE 6.S4: **Mutations detected in *cacna2d4a* F1 CRISPR fish.** Mutations found in 24 individual F1 fish obtained by outcrossing injected fish to wt are shown. +/- numbers indicate size of insertions and/or deletions. Numbers in brackets to the very right represent the number of mutant sequences found to the total number of clones sequenced. When only wild type sequences were found, the fish was declared as wild type. However, there is a chance that some of these "wild type" fish actually carry mutations that were not detected due to small number of clones sequenced.

***cacna2d4b* CRISPR F1 genotypting**

wt	AAGATCTCGTGATTGTGGTGGATGTGAGCGGCAGCATGAAGGGCCTAAAAATGACCATCGCCAAA		
F1#1	AAGATCTCGTGATTGTGGTGGATGTGAGCGGCA-TGTGAAGGGCCTAAAAATGACCATCGCCAAA	-1 (-3+2)	(4/6)
F1#2	AAGATCTCGTGATTGTGGTGGATGTGAGCGGCA-TGTGAAGGGCCTAAAAATGACCATCGCCAAA	-1 (-3+2)	(4/5)
F1#3	AAGATCTCGTGATTGTGGTGGATGTGAGCGGCA-TGTGAAGGGCCTAAAAATGACCATCGCCAAA	-1 (-3+2)	(4/7)
F1#4	AAGATCTCGTGATTGTGGTGGATGTGAGCC---------------TAAAAATGACCATCGCCAAA	-15	(3/6)
F1#5	AAGATCTCGTGATTGTGGTGGATGTGAG---------------CCTAAAAATGACCATCGCCAAA	-15	(1/3)
F1#6	AAGATCTCGTGATTGTGGTGGATGTGA------------AGGGCCTAAAAATGACCATCGCCAAA	-12	(1/3)
F1#7	wt		(0/3)
F1#8	wt		(0/3)
F1#9	AAGATCTCGTGATTGTGGTGGATGTGA------------AGGGCCTAAAAATGACCATCGCCAAA	-12	(2/3)
F1#10	AAGATCTCGTGATTGTGGTGGATGTGAGCGGCCTAAAAATGAGTGGATAAAAATGACCATCGCCAAA	+2 (-13+15)	(2/3)
F1#11	AAGATCTCGTGATTGTGGTGGATGTGAGCGGCCTAAAAATGAGTGGATAAAAATGACCATCGCCAAA	+2 (-13+15)	(2/3)
F1#12	wt		PCRseq
F1#13	AAGATCTCGTGATTGTGGTGGATGTGAGCGGCA-TGTGAAGGGCCTAAAAATGACCATCGCCAAA	-1 (-3+2)	(1/3)
F1#14	wt		PCRseq
F1#15	AAGATCTCGTGATTGTGGTGGATGTGA------------AGGGCCTAAAAATGACCATCGCCAAA	-12	(2/4)
F1#16	AAGATCTCGTGATTGTGGTGGATGTGAGCGGCCTAAAAATGAGTGGATAAAAATGACCATCGCCAAA	+2 (-13+15)	(1/3)
F1#17	AAGATCTCGTGATTGTGGTGGATGTGA------------AGGGCCTAAAAATGACCATCGCCAAA	-12	(2/3)

FIGURE 6.S5: **Mutations detected in *cacna2d4a* F1 CRISPR fish.** Mutations found in 17 individual F1 fish obtained by outcrossing injected fish to wt are shown. For explanation, see caption of Supplementary Figure 6.S4.

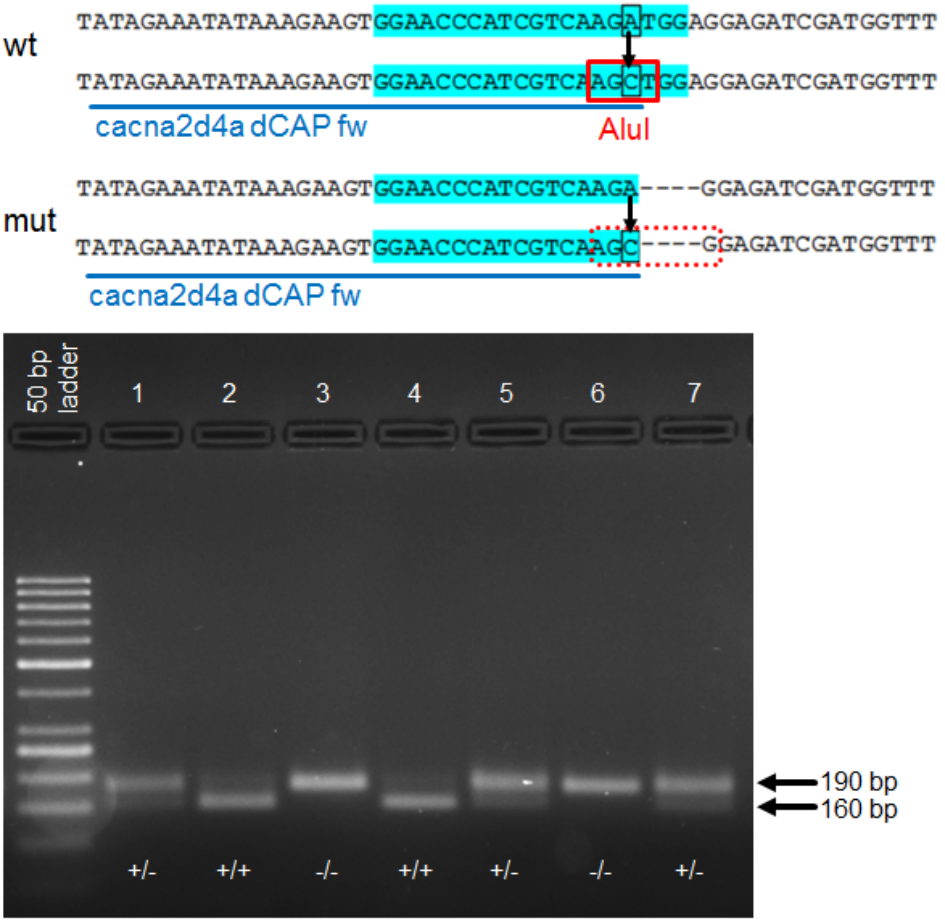

FIGURE 6.S6: **Genotyping strategy for the *cacna2d4a* mutant line, carrying a 4 bp deletion.** A dCAPs forward primer was designed, introducing an AluI restriction site exclusively in the wt, but not the mutant allele **(top)**. Amplification with cacna2d4a_ dCAP_ fw and cacna2d4a_ dCAP_ rev primers (Table 6.1) results in a 190 bp PCR product that in the wt condition is cut in two fragments of 160 and 35 bp. An example of genotyping F2 fish obtained from incrossing heterozygous F1 fish is shown **(bottom)**. Homozygous mutants exhibit one band of 190 bp, wt fish one band of 160 bp and heterozygous mutants bands of both sizes. The 35 bp fragment cannot be seen on the gel.

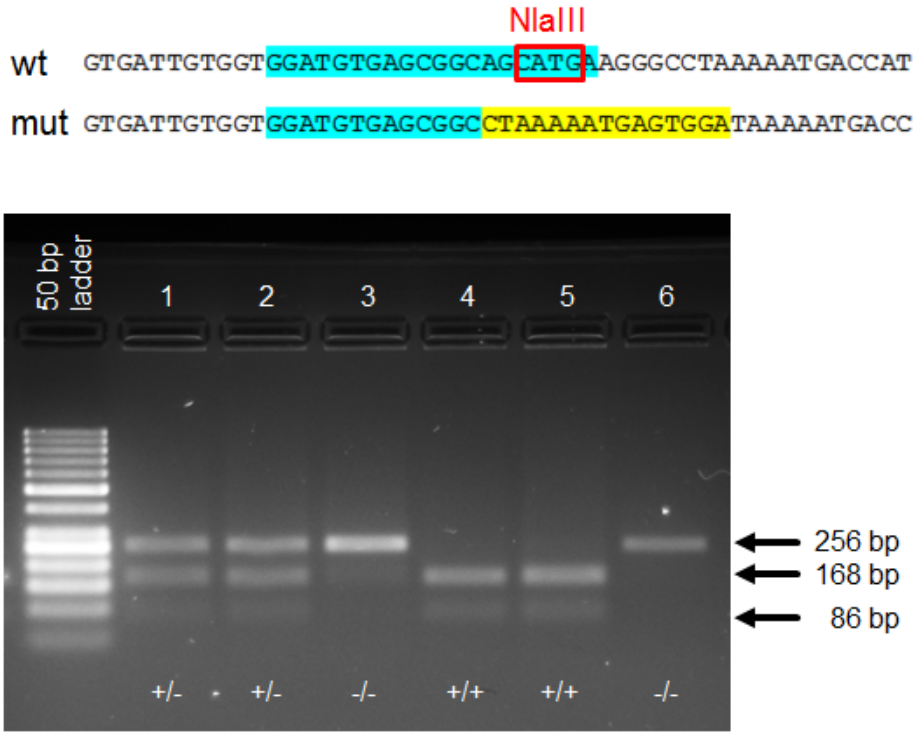

FIGURE 6.S7: **Genotyping strategy for the *cacna2d4b* mutant line, carrying a 2 bp insertion.** An NlaIII restriction site is removed in the mutated sequence **(top)**. Amplification with cacna2d4b_ CRISP2_ fw2 and cacna2d4b_ CRISP2_ rev primers (Table 6.1) results in a 256 bp PCR product, which in wt condition is cut in two fragments (168 and 86 bp). An example of genotyping F2 fish obtained from incrossing heterozygous F1 fish is shown **(bottom)**. Homozygous mutants exhibit one band of 256 bp, wt fish two bands of 168 bp and 86 bp, and heterozygous mutants bands of all 3 sizes.

Chapter 7

General Discussion

In the studies presented here, we have used the zebrafish model to elucidate retinal function from different perspectives. In order to gain a better understanding of retinal processing on the cellular level, we have attempted to transgenically label different subtypes of bipolar cells, known to provide parallel channels for different properties of a visual stimulus.

In course of this work, we found genes encoding Ca^{2+} binding proteins CaBP2 and CaBP5 to be specifically expressed in bipolar cells of larval and adult zebrafish, likely contributing to retinal signaling. Such a role might be executed by regulating voltage-activated Ca^{2+} channels at retinal ribbon synapses.

One transgenic construct driving expression in retinal bipolar cells harbors regulatory regions of the *mglur6b* gene, known to be involved in the ON bipolar cell response. By investigating transgene expression in this *mglur6b:EGFP* transgenic line in detail, we were able to extend the knowledge of *mglur6b* expression to cell types such as olfactory bulb projection neurons, suggesting novel functions of mGluR6 signaling in the brain.

Photoreceptor and bipolar cell ribbon synapses ensure tonic neurotransmitter release and modulate release in a graded manner in response to changes in light intensities. Mutation of genes encoding Ca^{2+} channel subunits localizing to retinal ribbon synapses are known to cause retinal dysfunction. We have examined the role of auxiliary $\alpha_2\delta_4$ subunits of voltage-gated Ca^{2+} channels, shown to be crucial for cone function in humans, in the zebrafish retina. Our results suggest conserved roles of genes encoding $\alpha_2\delta_4$ in trafficking of Ca^{2+} channel complexes to retinal synapses, validating zebrafish as model for cone dysfunction caused by *CACNA2D4* mutation.

An underlying aspect of all the studies presented here, especially important when comparisons to mammalian species are made, is the impact of teleost-specific genome duplication on gene evolution. The following sections will integrate and discuss results from our studies, and point out future directions.

7.1 The fates of duplicated genes involved in retinal signaling

Whole genome duplication (WGD) has been recognized as a major facilitator for generating organismal complexity and diversification of evolutionary lineages. One such event took place at the base of all extant teleost fishes, and is termed teleost-specific WGD (TS-WGD). WGD results in doubling of all genes of an organism. Duplicated gene pairs, also called paralogues, are initially functionally redundant and thus released from selective constraint, making them amenable to different evolutionary scenarios. Briefly, one duplicate may be lost (non-functionalization), functions of the ancestral gene may become divided between the duplicates (subfunctionalization), or one duplicate may acquire novel functions (neofunctionalization). Most gene pairs resulting from TS-WGD have lost one paralogue during evolution of extant teleost species. Only up to 20% of TS-WGD duplicated genes are estimated to have been retained in the lineage leading to zebrafish (Postlethwait et al., 2004; Postlethwait et al., 2000; Woods et al., 2005). Therefore, it is striking that all zebrafish genes investigated in this thesis (*mglur6b*, *eaat5b*, *cabp2a*, *cabp2b*, *cabp5a*, *cabp5b*, *cacna2d4a* and *cacna2d4b*) are duplicates retained after TS-WGD, strongly supported by branching patterns in phylogenetic trees and positioning of paralogues on different chromosomes (Di Donato et al., 2013; Gesemann et al., 2010; Haug et al., 2013) (and Chapter 6, page 101).

It has been shown that some classes of genes have been preferentially retained after WGD events (see Chapter 2.4.8, page 28). The functional category suggested to be most enriched after TS-WGD is genes involved in ion channel and transporter activity (Kassahn et al., 2009). The protein products of the genes investigated in the studies presented here largely fall into this category, with Cacna2d4 proteins being Ca^{2+} channel subunits, CaBPs associating with Ca^{2+} channels, EAAT5 constituting a transporter with intrinsic Cl^- channel properties, and the glutamate receptor mGluR6 controlling cation channel closure. Also other studies of protein families, with members functioning as ion channels, have revealed numerous retained duplicates derived from TS-WGD (Kastenhuber et al., 2013; Niederhausern et al., 2013). Why the category of ion channels/transporters and associated proteins has been over-retained is still not known. However, our findings support the notion that such genes, especially important for neuronal signaling, have been over-retained after TS-WGD.

A fate frequently acquired by retained duplicate gene pairs is subfunctionalization due to complementary degenerative mutation in gene regulatory regions (Force et al., 1999). This leads to distribution of ancestral expression domains onto the two WGD paralogues. Neofunctionalization is much less frequent due to the low rate that beneficial mutations occur at. Like subfunctionalization, also neofunctionalization can take place at the level of gene regulatory regions, giving rise to novel expression domains. Gene expression patterns of TS-WGD derived paralogues can therefore give us insights into their evolutionary history.

Among the genes investigated, paralogous *cabp2a* and *cabp2b* genes show expression patterns strongly suggesting subfunctionalization. Mammalian *cabp2* is expressed both in the retina and in auditory hair cells (Cui et al.,

2007; Nakajima et al., 2009), and this pattern seems to be divided upon *cabp2a* and *cabp2b* in zebrafish larvae, being espressed in the retinal inner nuclear layer (INL) and in auditory hair cells, respectively. One has to add that *cabp2b* is also found in the INL, although only at very low levels (see Chapter 3, on page 45). Similarly, *cacna2d4a* and *cacna2d4b* seem to have undergone subfunctionalization in the larval retina, where they are almost exclusively expressed in the INL and in photoreceptors, respectively (see Chapter 6, page 101). Interestingly, expression patterns of these paralogues, suggesting subfunctionalization in larvae, become more similar to their respective paralogue in the adult retina.

For the *cabp5* and *mglur6* paralogues, retinal expression patterns do not indicate subfunctionalization. Both *mglur6* paralogues are expressed in INL and ganglion cell layer (GCL) (Huang et al., 2012), and both *cabp5* paralogues in the INL (see Chapter 3, on page 45). It is however highly unlikely that these paralogues carry out redundant functions, unless gene-dosage effects are responsible for their retention (see Chapter 2.4.5, page 24). Alternatively, subfunctionalization might have occurred between subtypes of retinal cells, something that is difficult to resolve by chromogenic RNA *in-situ* hybridization. Since many genes expressed in the retina have apparently been retained as duplicates, and at the same time, teleosts have a higher number of retinal cell subtypes (see Chapter 1, page 1), it is even conceivable that TS-WGD has contributed to this expansion of retinal cell types. Another alternative explanation is that subfunctionalization might have not occurred at the level of transcriptional regulation, but at the level of coding sequence. For example, proteins encoded by *cabp* TS-WGD duplicates might have reciprocally lost sites for protein-protein interactions, sub-dividing their function onto different pathways transducing Ca^{2+} signals.

Instances of neofunctionalization are generally rare (see Chapter 2.4.4, page 23), and also in the genes investigated we could not find clear examples of neofunctionalization. *cacna2d4b* expression in the cerebellum may be an example of neofunctionalization, since cerebellar expression has not been reported from mammals. However, mammalian *Cacna2d4* expression in the brain is still ambiguous (De Sevilla Muller, Luis Perez et al., 2013; Qin et al., 2002; Schlick, Flucher, and Obermair, 2010). Additionally and importantly, mammalian expression patterns do not necessarily represent the ancestral state preceding TS-WGD. Expression data from intermediate species are needed to gain more insight into duplicate gene evolution. Of particular importance will be extant species closely related to the fish ancestor that underwent TS-WGD. The spotted gar (*Lepisosteus aculeatus*), belonging to the group of basal ray-finned fishes that have not undergone TS-WGD, is such an animal. With its recently sequenced and slowly evolving genome (Braasch et al., 2016), and the ability to be kept in the lab, the gar is a species much needed for bridging the evolution of gene regulation between mammals and zebrafish.

Since subfunctionalization, simply dividing ancestral gene function between duplicates, is the most frequent scenario for evolution of duplicate genes retained after TS-WGD, ancestral gene functions appear to stay frequently conserved in the duplicates. This is important when translating insights gained from zebrafish to non-teleost vertebrates, for example when using zebrafish as model for neurological diseases. However, in order to

model full loss-of function phenotypes of mammalian genes, double knockouts of paralogous zebrafish genes might be required in some cases.

7.2 Ca^{2+} signaling at retinal ribbon synapses

In the studies presented in this thesis, we have investigated genes suggested to be involved in neurotransmission at retinal ribbon synapses, i.e. members of the CaBP family (Chapter 3, page 45 and Chapter 4 on page 55) and subunits of voltage-gated Ca^{2+} channel subunits (Chapter 6, page 101).

Ribbon synapses are found in cells of the nervous system required to modulate vesicle release in a graded manner, opposed to all-or-nothing signaling at conventional synapses. This is the case for the sensory cells of the visual and auditory system, as well as the vestibular organ, electroreceptive organs and the lateral line system. In addition to sensory cells, also bipolar cells exhibit ribbon synapses and transmit graded signals.

Like at conventional synapses, exocytosis at ribbon synapses is driven by voltage-gated Ca^{2+} channels, which are located at the plasma membrane in close proximity to the ribbons. At conventional synapses, Ca^{2+} dependent inactivation (CDI) of voltage-gated Ca^{2+} channels prevents prolonged Ca^{2+} influx. CDI is a result of ubiquitously expressed Calmodulin binding to Ca^{2+} channels and inactivating them upon Ca^{2+} binding (reviewed by Simms and Zamponi, 2014). Since the ability to mediate sustained vesicle release is demanded from voltage-gated Ca^{2+} channels at ribbon synapses, mechanism abrogating CDI are required.

Cacna1f is an exceptional member of pore-forming α_1 subunits of voltage-gated Ca^{2+} channels, expressed in photoreceptors, since it lacks CDI (McRory et al. 2004) due to an autoinhibitory domain that occludes Calmodulin binding sites essential for CDI (Wahl-Schott et al., 2006; Singh et al., 2006). Also the α_1 subunit Cacna1d is expressed in the retina, with strong signal in the INL and a subset of GCL neurons, and weaker expression in photoreceptors (Kersten, Ferry F J et al., 2010), and is a good candidate for driving vesicle exocytosis from bipolar cell terminals. Although Cacna1d does not possess an autoinhibitory domain like Cacna1f, CDI can be precluded by Ca^{2+}-binding proteins (CaBPs). More specifically, it has been shown that CaBP4 counteracts binding of Calmodulin to Cacna1d (Yang et al., 2006), probably by an allosteric mechanism (Yang, Johny, and Yue, 2014), and also CaBP1 binding abrogates CDI of Cacna1d (Cui et al., 2007). These interactions are thought to be meaningful for inhibiting CDI in auditory hair cells, where Cacna1d, CaBP1 and CaBP4 are co-expressed (Cui et al., 2007). However, genes encoding neither CaBP4 nor CaBP1 have been proven to be expressed in bipolar cells of mammals or zebrafish (Di Donato et al., 2013; Haeseleer et al., 2004; Haeseleer et al., 2000), opening the possibility that inhibiting CDI in bipolar cells might be conveyed by other CaBPs.

Mouse retinal bipolar cells express CaBP5 (Haeseleer et al., 2000), and probably also CaBP2 (Nakajima et al., 2009). Our expression analysis of zebrafish *cabp2a*, *cabp2b*, *cabp5a* and *cabp5b* genes, (Chapter 3, page 45) together with the study by Di Donato et al., (2013) shows expression of all these genes in bipolar cells of the developing and the adult zebrafish retina, suggesting conserved expression patterns of genes encoding CaBP2 and

CaBP5 between zebrafish and mouse. Due to these expression patterms, it is conceivable that in bipolar cells of mammals as well as zebrafish, CaBP2 and/or CaBP5 proteins adopt a similar role to the proven function of CaBP4 in auditory hair cells. In support of this, moderate inhibition of Cacna1d CDI by CaBP5 was shown when expressed together in HEK cells (Cui et al., 2007). The retinae of Cabp5 knockout mice show decreased light sensitivity, measured in retinal ganglion cells (Rieke, Lee, and Haeseleer, 2008). The electroretinogram however appeared normal, supporting a role of CaBP5 in the inner retina. In summary, it is conceivable that CaBP5 tunes neurotransmitter release from bipolar cell synapses by regulating voltage-gated Ca^{2+} channels.

While CaBP5 function in the retina has been addressed, no reports on CaBP2 function in the mouse retina exist, except for one Microarray study suggesting Cabp2 expression in ON bipolar cells (Nakajima et al., 2009). However, a role of CaBP2 in the auditory system has been recognized, exerted at least in part by regulation of Cacna1d (Schrauwen et al., 2012).

Almost certainly, CaBPs expressed in the retina also have other functions than modulating CDI of voltage-gated Ca^{2+} channels. Despite the fact that CDI is regarded intrinsic to Cacna1f, CaBP4 causes it to activate at more hyperpolarized membrane potentials (Haeseleer et al., 2004). Moreover, CaBP5 has been suggested to directly act in synaptic vesicle exocytosis by interacting with Munc-19-1 and Myosin VI (Sokal and Haeseleer, 2011), which are both players in the synaptic vesicle cycle. Also the fact that CaBP5 not only localizes to bipolar cell axon terminals, but is also found in the cell body (Haeseleer et al., 2000), suggests CaBP5 functions in addition to modulating neurotransmitter release at the synaptic terminal.

Since genes encoding both CaBP2 and CaBP5 are very likely expressed in bipolar cells of mammals and zebrafish, it is tempting to speculate that they might differentially modulate Ca^{2+} dynamics in bipolar cell terminals, ultimately contributing to shaping the bipolar cell output. If different CaBPs were differentially expressed by bipolar cell subtypes, they could potentially provide a mechanism for differentially tuning their output in response to a visual stimulus. The duplicated genes encoding CaBPs in zebrafish might permit even more diverse Ca^{2+} signals than the mammalian CaBP repertoire.

To test whether CaBP2 and CaBP5 proteins indeed shape bipolar cell output in a subtype-specific manner, knowledge about bipolar cell subtype-specific expression is required. This would be greatly facilitated by antibodies specifically recognizing zebrafish CaBP2 and CaBP5 proteins. Alternatively, fluorescent *in situ* hybridization in combination with markers for bipolar cell subpopulations, such as PKCα or transgenically labelled bipolar cell subtypes (Chapter 4, page 55 and Chapter 5, page 73) can resolve bipolar cell subtype-specific expression in zebrafish. To ultimately test the contribution to shaping the bipolar cell signal, knockout of genes encoding CaBP2 and CaBP5 proteins are required. Stable knockout of virtually any gene is now possible in zebrafish due to the establishment of efficient genome editing by CRISPR/Cas9 (Hwang et al., 2013). Ca^{2+} (Dreosti et al., 2009; Baden et al., 2013; Yonehara et al., 2013) and glutamate imaging (Marvin et al., 2013; Borghuis et al., 2013) of bipolar cell terminals in such knockout lines provide excellent tools for studying the effects of CaBPs on the bipolar cell output.

7.3 The role of auxiliary $\alpha_2\delta_4$ Ca^{2+} channel subunits in retinal function

Like CaBPs, $\alpha_2\delta_4$ subunits are interactors of pore-forming voltage-gated Ca^{2+} channel subunits. In Chapter 6 (page 101) we have investigated one such auxiliary Ca^{2+} channel subunit with proven function in the retina. Mutations in *CACNA2D4A*, encoding $\alpha_2\delta_4$ (or CACNA2D4), cause cone dysfunction in human patients (Wycisk et al., 2006b; Ba-Abbad et al., 2016; Vincent et al., 2014; Huang et al., 2015), and studies on mouse models have given first imortant insights into the mechanisms leading to these defects (Wycisk et al., 2006a; Ruether et al., 2000; Caputo et al., 2015). Using the cone-dominant zebrafish, we are aiming to gain a better understanding of disease onset and progression. Zebrafish possess two orthologues of human *CACNA2D4*, termed *cacna2d4a* and *cacna2d4b*, which both show basically conserved expression patterns to their mammalian orthologue, validating zebrafish as a model to study CACNA2D4 function in a first step. We have used CRISPR/Cas9 genome editing to generate *cacna2d4a* and *cacna2d4b* mutant lines carrying truncating frameshift mutations early on in the reading frames of the respective paralogues. Interestingly, the *cacna2d4b* mutant line showed strongly decreased expression of Cacna1fa in photoreceptor terminals. This is very likely a direct effect of Cacna2d4b binding to Cacna1fa, since direct interaction of orthologous Cacna2d4 and Cacna1f proteins has been shown in the mouse retina (Lee et al., 2015). Furthermore, the synaptic phenotype of Cacna1fa mislocalization in *cacna2d4b* mutant zebrafish was very specific. No other presynaptic or postsynaptic protein investigated showed mislocalization or decreased abundance in our mutant lines at 5 days post fertilization, the developmental stage when the zebrafish retina has just become fully functional and the only time point analyzed so far. Curiously, human patients carrying *CACNA2D4* mutations mostly show decreased visual acuity only during adulthood (Wycisk et al., 2006b; Huang et al., 2015; Ba-Abbad et al., 2016), while photophobia was sometimes noted already during infancy (Wycisk et al., 2006b; Vincent et al., 2014). Owing to this successive appearance of symptoms in these patients, it is likely that initially subtle synaptic defects become more severe during adulthood, in some cases even leading to cone dystrophy (Wycisk et al., 2006b). Thus, specific mislocalization of Cacna1fa in our *cacna2d4b* mutant zebrafish line provides a very good starting point for follow-up examination of synaptic proteins, retinal morphology and function in further developmental stages, up to the adult and also in the ageing retina. Since *cacna2d4a* and *cacna2d4b* are co-expressed in some retinal cell types during adulthood, the additional examination of double mutant lines will probably be advantageous in studying the disease phenotype.

Expression patterns of zebrafish *cacna2d4* genes in photoreceptors and cells of the INL, among them very likely bipolar cells, suggest that the role of Cacna2d4 proteins is not restricted to photoreceptors. Similarly, *Cacna2d4* transcripts were also found in the INL of the mouse retina (Postel et al., 2013), suggesting conservation of Cacna2d4 function also in this retinal layer. Bipolar cell axon terminals of Cacna2d4 mutant mice have abnormal ribbon synapses, exhibiting spherical instead of elongated shape (Ruether et al., 2000). It will therefore be interesting to see whether also α_1

subunits expressed in bipolar cell axon terminals show mislocalization due to cacna2d4 mutation. One $\alpha 1$ subunit worth investigating, and for which also a zebrafish-specific antibody has been generated (Sidi et al. 2004), is Cacna1da, encoded by a gene expressed in the INL of both larval (Sidi et al., 2004) and adult zebrafish (unpublished observation).

The role of auxiliary $\alpha_2\delta$ subunits targeting pore-forming α_1 subunits to the plasma membrane is well established.

It has also been shown that $\alpha_2\delta$ subunits can influence single channel properties of Ca^{2+} channels (reviewed by Dolphin, 2013). In an *in vitro* study, Cacna1f associated with Cacna2d4 showed weaker voltage dependent activation than when associated with Cacna2d1 (Lee et al., 2015). Thus, modulation of voltage dependence by Cacna2d4 proteins might play a role in shaping the photoreceptor and bipolar cell signal. However, since decrease of Cacna1fa due to *cacna2d4b* mutations was so profound in our study, altered single-channel properties very probably only marginally contribute to cone dysfunction, if at all.

It is not clear yet at which point α_1 trafficking is dependent on $\alpha_2\delta$. It has been found that the MIDAS motif, which is part of the Van Willebrand Factor A (WVA) domain contained in all $\alpha_2\delta$ subunits, is crucial for this role (Canti et al., 2005; Hoppa et al., 2012). Mutation of the MIDAS motif in $\alpha_2\delta_2$ abolished its trafficking function and caused α_1 subunits to be retained in intracellular compartments of undetermined identity (Canti et al., 2005). Interestingly, we found *cacna2d4b* mutation to cause ectopic localization of Cacna1fa in photoreceptors. It is a possibility that the ectopic punctate staining found outside the synaptic compartment represents Cacna1fa retained in certain cellular compartments, for example the Golgi. Alternatively, Cacna1fa might mislocalize to the plasma membrane and eventually form ectopic synapses with second-order bipolar cells, since such synapses are commonly found in photoreceptor dysfunction (Specht et al., 2007; Zabouri and Haverkamp, 2013; Liu et al., 2013). It will be interesting to determine the exact location of ectopic Cacna1fa clusters. Super-resolution microscopy together with co-labeling of organelles like the Golgi should make it possible to resolve subcellular localization, possibly revealing the point of $\alpha_2\delta$ action in Ca^{2+} channel complex trafficking.

7.4 Conclusion

In summary, our studies have shown that the zebrafish model is ideally suited for studying molecular and cellular aspects of retinal function. Besides conserved retinal structure, gene expression patterns studied in this thesis were also found to be well conserved between the retina of mammals and zebrafish. In addition, conserved retinal function of the disease gene *CACNA2D4* has been revealed, further validating zebrafish as model for studying dysfunctions of the human retina. Established methods available for readout of retinal function, together with CRISPR/Cas9 genome editing and newly emerging imaging techniques compose a powerful toolkit for future studies of retinal function and disease in the zebrafish model.

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Nomenclature

aa	amino acids
ac	anterior commissure
AC	amacrine cell
BC	bipolar cell
BPC	bipolar cell
CaBP	Ca^{2+}-binding protein
CCe	Corpus cerebelli
CDI	Ca^{2+} dependent inactivation
Ce	cerebellum
ChAT	acetylcholinesterase
CNE	conserved non-coding element
CP	cone pedicles
DDC	Duplication-Degeneration-Complementation
DDI	Duplication Degeneration Innovation
dpf	days post fertilization
Dr	*Danio rerio*
DT	dorsal thalamus
EAAT	excitatory amino acid transporter
EAC	escape from adaptive conflict
egpf	enhanced green fluorescent protein

EmT	eminentia thalami
GC	retinal ganglion cell
GCL	ganglion cell layer
GL	granule cell layer of CCe
H	hypothalamus
HC	horizontal cell
Hd	periventricular hypothalamus
Hs	*Homo sapiens*
Hv	ventral hypothalamus
iGluRs	Ionotropic glutamate receptors
INL	inner nuclear layer
IPL	inner plexiform layer
LOT	lateral olfactory tract
M3	migrated area of eminentia thalami
mGluRs	Metabotropic glutamate receptors
ML	molecular layer of CCe
Mm	*Mus musculus*
mya	million years ago
NFL	nerve fiber layer
OB	olfactory bulb
oc	optic chiasm
ON	optic nerve
OPL	outer plexiform layer
P	pallium
PCL	purkinje cell layer

PGZ	periventricular grey zone
Ph	photoreceptor layer
PTN	posterior tuberal nucleus
Ret	retina
rs	rod spherules
S	subpallium
Sd	dorsal division of subpallium
SFGS	superficial grey/fibrous layer of optic tectum
Tel	telencephalic neurons
TeO	optic tectum
TR	thalamic region
TS	teleost-specific
VTG	Vitellogenin
WGD	Whole genome duplication
wt	wild type
WVA	Van Willebrand Factor A

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Appendix A

CV

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2015	**Talk:** Investigating the role of calcium channel $\alpha_2\delta$-4 subunits in the zebrafish retina (2015). European Retina Meeting, Brighton, UK.
2014	**Poster presentation:** A genetic approach to elucidate visual processing in the zebrafish retina (2014). ARVO meeting, Orlando, Florida, USA.
2013	**Poster presentation:** Shedding light on visual processing in the zebrafish retina: a genetic approach (2013). European Zebrafish Meeting, Barcelona, Spain.
2011	**Talk:** Hydra myc genes and their involvement in ancestral signaling pathways (2011). International workshop on basal metazoans and their evolution, Tutzing, Germany.

List of publications

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